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(54) Title: **IMPROVED VACCINES**

(57) Abstract: The invention refers to an improved vaccine against infections with pathogens, especially viral pathogens, comprising an antigen, a peptide of the formula R₁-XZX_N-XZX-R₂ and an immunostimulatory deoxynucleic acid containing deoxyinosine and/or deoxyuridine residues.



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Improved Vaccines

The present invention relates to improved vaccines, especially viral vaccines and methods of making thereof.

Host protection from invading pathogens involves cellular and humoral effectors and results from the concerted action of both non-adaptive (innate) and adaptive (acquired) immunity. The latter is based on specific immunological recognition mediated by receptors, is a recent acquisition of the immune system, and is present only in vertebrates. The former evolved before the development of adaptive immunity, consisting of a variety of cells and molecules distributed throughout the organism with the task of keeping potential pathogens under control.

B and T lymphocytes are the mediators of acquired antigen-specific adaptive immunity, including the development of immunological memory, which is the main goal of creating a successful vaccine. Antigen presenting cells (APCs) are highly specialized cells that can process antigens and display their processed fragments on the cell surface together with molecules required for lymphocyte activation. This means that APCs are very important for the initiation of specific immune reactions. The main APCs for T lymphocyte activation are dendritic cells (DCs), macrophages, and B cells, whereas the main APCs for B cells are follicular dendritic cells. In general DCs are the most powerful APCs in terms of initiation of immune responses stimulating quiescent naive and memory B and T lymphocytes.

The natural task of APCs in the periphery (e.g. DCs or Langerhans cells) is to capture and process antigens, thereby being activated they start to express lymphocyte co-stimulatory molecules, migrate to lymphoid organs, secrete cytokines and present antigens to different populations of lymphocytes, initiating antigen-specific immune responses. They not only activate lymphocytes, under certain circumstances, they also tolerize T cells to antigens.

Antigen recognition by T lymphocytes is major histocompatibility complex (MHC)-restricted. A given T lymphocyte will recognize an antigen only when the peptide is bound to a particular MHC

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molecule. In general, T lymphocytes are stimulated only in the presence of self MHC molecules, and antigen is recognized only as peptides bound to self MHC molecules. MHC restriction defines T lymphocyte specificity in terms of the antigen recognized and in terms of the MHC molecule that binds its peptide fragment.

Intracellular and extracellular antigens present quite different challenges to the immune system, both in terms of recognition and of appropriate response. Presentation of antigens to T cells is mediated by two distinct classes of molecules ~ MHC class I (MHC-I) and MHC class II (MHC-II), which utilize distinct antigen processing pathways. Mainly one could distinguish between two major antigen processing pathways that have evolved. Peptides derived from intracellular antigens are presented to CD8⁺ T cells by MHC class I molecules, which are expressed on virtually all cells, while extracellular antigen-derived peptides are presented to CD4⁺ T cells by MHC-II molecules. However, there are certain exceptions to this dichotomy. Several studies have shown that peptides generated from endocytosed particulate or soluble proteins are presented on MHC-I molecules in macrophages as well as in dendritic cells. Therefore APCs like dendritic cells sitting in the periphery, exerting high potency to capture and process extracellular antigens and presenting them on MHC-I molecules to T lymphocytes are interesting targets in pulsing them extracellularly with antigens in vitro and in vivo.

The important and unique role of APCs, including stimulating activity on different types of leukocytes, is reflecting their central position as targets for appropriate strategies in developing successful vaccines. Theoretically one way to do so is to enhance or stimulate their natural task, the uptake of antigen(s). Once pulsed with the appropriate antigens the vaccine is directed against, APCs should start to process the uptaken antigen(s), thereby being activated, expressing lymphocyte co-stimulatory molecules, migrating to lymphoid organs, secreting cytokines and presenting antigens to different populations of lymphocytes thereby initiating immune responses.

Activated T cells generally secrete a number of effector cytokines in a highly regulated fashion, e.g. interleukin.2 (IL-

2), IL-4, IL-5, IL-10 and interferon-gamma (IFN-g). The functional detection of cytotoxic T lymphocyte responses to specific antigens (e.g. tumor antigens, in general antigens administered in a vaccine) is commonly monitored by an ELISpot assay (enzyme-linked immunospot assay), a technique analyzing cytokine production at the single cell level. In the present invention an ELISpot assay for the cellular immunity (type 1 immune response) promoting cytokine IFN-g is used to monitor successful antigen-specific T cell activation. Furthermore, the cytokine IL-4 is determined as an indicator for a type 2 response, usually involved in promoting strong humoral responses. In addition, the humoral immune response was determined by ELISA (IgG1 as indicator for a type 2 response, IgG2b as indicator for a type 1 response).

It has previously been shown that polycations efficiently enhance the uptake of MHC class I-matched peptides into tumor cells, a peptide or protein pulsing process which was called "TRANSloading". Furthermore, it has been shown that polycations are able to "TRANSload" peptides or proteins into antigen presenting cells in vivo as well as in vitro. In addition, co-injection of a mixture of poly-L-arginine or poly-L-lysine together with an appropriate peptide as a vaccine protects animals from tumor growth in mouse models. This chemically defined vaccine is able to induce a high number of antigen/peptide-specific T cells. That was shown to be at least partly attributable to an enhanced uptake of peptides into APCs mediated by the polycation indicating that APCs when pulsed in vivo with antigens can induce T cell-mediated immunity to the administered antigen.

As opposed to adaptive immunity, which is characterised by a highly specific but relatively slow response, innate immunity is based on effector mechanisms that are triggered by differences in the structure of microbial components relative to the host. These mechanisms can mount a fairly rapid initial response, which mainly leads to neutralization of the noxious agents. Reactions of innate immunity are the only defence strategy of lower phyla and have been retained in vertebrates as a first line host defence before the adaptive immune system is mobilised.

In higher vertebrates the effector cells of innate immunity are neutrophils, macrophages, and natural killer cells and probably also dendritic cells, whereas the humoral components in this pathway are the complement cascade and a variety of different binding proteins.

A rapid and effective component of innate immunity is the production of a large variety of microbicidal peptides with a length of usually between about 12 and about one hundred amino acid residues. Several hundred different antimicrobial peptides have been isolated from a variety of organisms, ranging from sponges, insects to animals and humans, which points to a widespread distribution of these molecules. Antimicrobial peptides are also produced by bacteria as antagonistic substances against competing organisms.

Two major subsets of CD4⁺ T cells (T-helper 1 (Th1) and T-helper 2 (Th2)) have been identified in mouse and human, based on their secretion of different cytokine profiles and their different effector functions. Th1 cells are mainly involved in the generation of so called type 1 immune responses, which are typically characterised by the induction of delayed-type hypersensitivity responses, cell-mediated immunity, immunoglobulin class switching to IgG2a/IgG2b and secretion of i.a. Interferon-gamma. In contrast, Th2 cells are involved in the generation of so called type 2 responses, which are characterised by the induction of humoral immunity by activating B cells, leading to antibody production including class switching to IgG₁ and IgE. Type 2 responses are also characterized by the secretion of the following cytokines: IL-4, IL-5, IL-6 and IL-10.

In most situations, the type of response induced (type 1 or type 2) has a significant impact on the protective efficacy of a vaccine. Alternative adjuvants tend to favour specific types of responses. However, adjuvant selection is complicated by functional unpredictabilities and also by commercial constraints and availability.

Infections with Influenza virus belong to the most important and frequent infections and has a significant mortality rate, especially for older people or people with deficiencies in the immune system. Currently, there are a number of Influenza vaccines on the market; however, not all vaccinations lead to protectivity against Influenza infections. Therefore, a need to improve current Influenza vaccines exists in order to enlarge the protection efficacy.

Moreover, since most of the current vaccines are almost exclusively eliciting type 2 responses, also a need exists to provide improved vaccines which show a type 1 directed immune response or vaccines which allow - in addition to a type 2 response - also a significant type immune reaction. Moreover, vaccines already available should be provided in an improved form which allows the induction of a type 1 response.

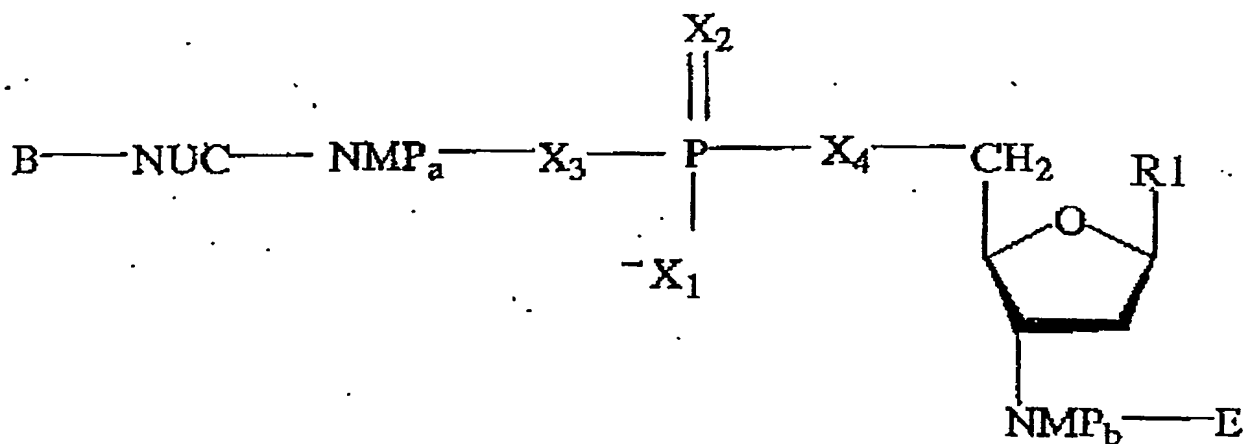
Therefore, the present invention provides an improved vaccine against (viral) infections comprising an antigen, a peptide of the formula $R_1\text{-XZXZ}_N\text{XZX-R}_2$ and an immunostimulatory deoxynucleic acids containing deoxyinosine and/or deoxyuridine residues.

According to the experiments performed in course of the present invention, the combination of these two types of Immunizers has shown a synergistical effect with respect to antigens. This was specifically shown with respect to common Influenza antigens (especially haemagglutinin and neuraminidase) and Hepatitis virus antigens. This synergistic effect especially for viral antigens was not derivable from the known properties of these substance classes. Although each of these two substance classes is known to have excellent immunostimulating properties (WO 02/32451, WO 01/93905 and PCT/EP02/05448), the combined effect for viral pathogens, especially for Influenza and Hepatitis virus antigens, was significantly better than could be expected from the mere addition of these single efficacies.

With the present invention it is also possible to significantly improve viral vaccines, especially Influenza or Hepatitis A, B or C vaccines, being already available or on the market simply by additionally providing the combination of the two types of Immunizers according to the present invention.

The present invention therefore provides a vaccine for preventing viral infections comprising

- an antigen, especially a viral antigen,
- a peptide comprising a sequence $R_1\text{-XZ}_N\text{XZ}_N\text{-R}_2$, whereby N is a whole number between 3 and 7, preferably 5, X is a positively charged natural and/or non-natural amino acid residue, Z is an amino acid residue selected from the group consisting of L, V, I, F and/or W, and R_1 and R_2 are selected independantly one from the other from the group consisting of -H , -NH_2 , -COCH_3 , -COH , a peptide with up to 20 amino acid residues or a peptide reactive group or a peptide linker with or without a peptide; X-R_2 may be an amide, ester or thioester of the C-terminal amino acid residue of the peptide (in the following also referred to as "Peptide A") and
- an immunostimulatory oligodeoxynucleic acid molecule (ODN) having the structure according to the formula (I)



wherein

R_1 is selected from hypoxanthine and uracile,
 any X is O or S,
 any NMP is a 2' deoxynucleoside monophosphate or monothiophosphate, selected from the group consisting of deoxyadenosine-, deoxyguanosine-, deoxyinosine-, deoxycytosine-, deoxyuridine-,

deoxythymidine-, 2-methyl-deoxyinosine-, 5-methyl-deoxycytosine-, deoxypseudouridine-, deoxyribosepurine-, 2-amino-deoxyribosepurine-, 6-S-deoxyguanine-, 2-dimethyl-deoxyguanosine- or N-isopentenyl-deoxyadenosine-monophosphate or -monothiophosphate,

NUC is a 2' deoxynucleoside, selected from the group consisting of deoxyadenosine-, deoxyguanosine-, deoxyinosine-, deoxycytosine-, deoxyinosine-, deoxythymidine-, 2-methyl-deoxyuridine-, 5-methyl-deoxycytosine-, deoxypseudouridine-, deoxyribosepurine-, 2-amino-deoxyribosepurine-, 6-S-deoxyguanine-, 2-dimethyl-deoxyguanosine- or N-isopentenyl-deoxyadenosine,

a and b are integers from 0 to 100 with the proviso that $a + b$ is between 4 and 150, and

B and E are common groups for 5' or 3' ends of nucleic acid molecules (in the following also referred to as "I-/U-ODN").

Of course, the present vaccine may further contain other substances, e.g. suitable pharmaceutically acceptable diluents or carrier, buffer or stabilising substances, etc..

The vaccine according to the present invention may further contain additional adjuvants, especially an $\text{Al}(\text{OH})_3$ adjuvant (Alum).

Alum, as meant herein includes all forms of Al^{3+} based adjuvants used in human and animal medicine and research. Especially, it includes all forms of aluminum hydroxide as defined in Römpp, 10th Ed. pages 139/140, gel forms thereof, aluminum phosphate, etc..

This is especially preferred for vaccines which are already on the market and contain such $\text{Al}(\text{OH})_3$ adjuvants. In such a case, the combination of Immunisers according to the present invention may simply be added to such an existing vaccine.

The present antigen is preferably a viral antigen. If pronounced (or exclusive) Th1 type 1 responses should be specifically necessary, T cell epitopes (see introduction above) are preferred as antigens. Preferably the antigen is a viral antigen. In the example section the present invention is proven

in principle and specifically effective with influenza and hepatitis viral antigens, namely with the hepatitis B surface antigen and hepatitis C antigens which are preferred antigens according to the present invention.

Of course, the pharmaceutical preparation may also comprise two or more antigens depending on the desired immune response. The antigen(s) may also be modified so as to further enhance the immune response.

Proteins or peptides derived from viral or bacterial pathogens, from fungi or parasites, as well as tumor antigens (cancer vaccines) or antigens with a putative role in autoimmune disease may be used as antigens (including derivatized antigens like glycosylated, lipidated, glycolipidated or hydroxylated antigens). Furthermore, carbohydrates, lipids or glycolipids may be used as antigens themselves. The derivatization process may include the purification of a specific protein or peptide from the pathogen, the inactivation of the pathogen as well as the proteolytic or chemical derivatization or stabilization of such a protein or peptide. Alternatively, also the pathogen itself may be used as an antigen. The antigens are preferably peptides or proteins, carbohydrates, lipids, glycolipids or mixtures thereof.

According to a preferred embodiment T cell epitopes are used as antigens. Alternatively, a combination of T cell epitopes and B cell epitopes may also be preferred.

Also mixtures of different antigens are of course possible to be used according to the present invention. Preferably, proteins or peptides isolated from a viral or a bacterial pathogen or from fungi or parasites (or their recombinant counterparts) are used as such antigens (including derivatized antigens or glycosylated or lipidated antigens or polysaccharides or lipids). Another preferred source of antigens are tumor antigens. Preferred pathogens are selected from human immunodeficiency virus (HIV), hepatitis A and B viruses, hepatitis C virus (HCV) or other Flaviviridae, such as Japanese encephalitis virus (JCV), rous sarcoma virus (RSV), Epstein Barr virus (EBV) Influenza virus, human papilloma virus (HPV), Rotavirus, Staphylococcus aureus,

Chlamydia pneumonias, Chlamydia trachomatis, Mycobacterium tuberculosis, Streptococcus pneumonias, Bacillus anthracis, Vibrio cholerae, Plasmodium sp. (Pl. falciparum, Pl. vivax, etc.), Aspergillus sp. or Candida albicans.

In the case of peptide antigens the use of peptide mimotopes/agonists/superagonists/antagonists or peptides changed in certain positions without affecting the immunologic properties or non-peptide mimotopes/agonists/superagonists/antagonists is included in the current invention. Peptide antigens may also contain elongations either at the carboxy or at the amino terminus of the peptide antigen facilitating interaction with the polycationic compound(s) or the immunostimulatory compound(s).

Antigens may also be derivatized to include molecules enhancing antigen presentation and targeting of antigens to antigen presenting cells.

The Influenza or Hepatitis antigen to be used according to the present invention is not generally restricted to a specific form, it seems that the effect according to the present invention is even further pathogen-specifically enhanced for Influenza, Hepatitis B or Hepatitis C, but not specific for a certain type of antigen from this Influenza or HBV pathogen. However, it is preferred to use the standard Influenza or HBV antigens also in the present vaccines, i.e. a haemagglutinin antigen, a neuraminidase antigen, a combined antigen or a combination of one or more of these antigens.

Preferably, proteins or peptides isolated from an Influenza virus, HBV or HCV source (e.g. a cell culture) or their recombinant counterparts are used as such antigens, including derivatized antigens.

The vaccine according to the present invention preferably further (or, specifically in the case of Influenza, HCV or HBV, even instead of the Peptide A) contains a polycationic peptide.

The polycationic peptides or compound to be used according to the present invention may be any polycationic compound which shows the characteristic effect according to the WO 97/30721.

Preferred polycationic compounds are selected from basic polypeptides, organic polycations, basic polyaminoacids or mixtures thereof. These polyaminoacids should have a chain length of at least 4 amino acid residues. Especially preferred are substances containing peptidic bounds, like polylysine, polyarginine and polypeptides containing more than 20%, especially more than 50% of basic amino acids in a range of more than 8, especially more than 20, amino acid residues or mixtures thereof. Other preferred polycations and their pharmaceutical compositions are described in WO 97/30721 (e.g. polyethyleneimine) and WO 99/38528. Preferably these polypeptides contain between 20 and 500 amino acid residues, especially between 30 and 200 residues.

These polycationic compounds may be produced chemically or recombinantly or may be derived from natural sources.

Cationic (poly)peptides may also be polycationic anti-bacterial microbial peptides. These (poly)peptides may be of prokaryotic or eukaryotic origin or may be produced chemically or recombinantly. Peptides may also belong to the class naturally occurring antimicrobial peptides. Such host defense peptides or defensives are also a preferred form of the polycationic polymer according to the present invention. Generally, a compound allowing as an end product activation (or down-regulation) of the adaptive immune system, preferably mediated by APCs (including dendritic cells) is used as polycationic polymer.

Furthermore, also neuroactive compounds, such as (human) growth hormone (as described e.g. in WO01/24822) may be used as immunostimulants (Immunizers).

Polycationic compounds derived from natural sources include HIV-REV or HIV-TAT (derived cationic peptides, antennapedia peptides, chitosan or other derivatives of chitin) or other peptides derived from these peptides or proteins by biochemical or recombinant production. Other preferred polycationic compounds are cathelin or related or derived substances from cathelicidin, especially mouse, bovine or especially human cathelicidins and/or cathelicidins. Related or derived cathelicidin substances contain the whole or parts of the

cathelicidin sequence with at least 15-20 amino acid residues. Derivations may include the substitution or modification of the natural amino acids by amino acids which are not among the 20 standard amino acids. Moreover, further cationic residues may be introduced into such cathelicidin molecules. These cathelicidin molecules are preferred to be combined with the antigen/vaccine composition according to the present invention. However, these cathelin molecules surprisingly have turned out to be also effective as an adjuvant for an antigen without the addition of further adjuvants. It is therefore possible to use such cathelicidin molecules as efficient adjuvants in vaccine formulations with or without further immunactivating substances.

The vaccine according to the present invention preferably contains as Peptide A KLKL₅KLK and as I-/U-ODN oligo d(IC)₁₃ (The combination of Peptide A and Oligo-d(IC)₁₃ is also referred as IC31). These two substances have shown specifically advantageous results in the experiments according to the present invention.

The vaccine according to the present invention may further (or, specifically in the case of Influenza, HCV or HBV, even instead of the U-/I-ODN) contain an oligodeoxynucleotide containing a CpG-motif as immunomodulating nucleic acids. The immunomodulating nucleic acids to be used according to the present invention can be of synthetic, prokaryotic and eukaryotic origin. In the case of eukaryotic origin, DNA should be derived from, based on the phylogenetic tree, less developed species (e.g. insects, but also others). In a preferred embodiment of the invention the immunogenic oligodeoxynucleotide (ODN) is a synthetically produced DNA-molecule or mixtures of such molecules. Derivates or modifications of ODNs such as thiophosphate substituted analogues (thiophosphate residues substitute for phosphate) as for example described in US patents US 5,723,335 and US 5,663,153, and other derivatives and modifications, which preferably stabilize the immunostimulatory composition(s) but do not change their immunological properties, are also included. A preferred sequence motif is a six base DNA motif containing an (unmethylated) CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines (5'-Pur-Pur-C-G-Pyr-Pyr-3'). The CpG motifs contained in the ODNs according to the present invention are more common in microbial than higher

vertebrate DNA and display differences in the pattern of methylation. Surprisingly, sequences stimulating mouse APCs are not very efficient for human cells. Preferred palindromic or non-palindromic ODNs to be used according to the present invention are disclosed e.g. in Austrian Patent applications A 1973/2000, A 805/2001, EP 0 468 520 A2, WO 96/02555, WO 98/16247, WO 98/18810, WO 98/37919, WO 98/40100, WO 98/52581, WO 98/52962, WO 99/51259 and WO 99/56755 all incorporated herein by reference. ODNs/DNAs may be produced chemically or recombinantly or may be derived from natural sources. Preferred natural sources are insects.

The vaccine according to the present invention may preferably contain a polycationic peptide and an oligodeoxynucleotide containing a CpG-motif in combination. In the course of the present invention it has even turned out that the combination of CpG-ODN and polycationic peptide shows improvement effects in Influenza vaccine compositions, which are comparable to the effects of the combination of Peptide A and I-/U-ODNs and cannot only be combined with Peptide A and I-/U-ODNs but even be used instead of them. Of course, also mixtures of different immunostimulatory nucleic acids (I-/U-ODNs, CpG-ODNs,...) and Peptide A variants (as well as other Immunizers) may be used according to the present invention.

According to another aspect, the present invention also relates to the use of a combination of Peptide A and a I-/U-ODN, both as defined according to the present invention, to improve the protective efficacy of a vaccine against a viral pathogen, especially influenza virus, HCV or HBV, HIV, HPV or JEV. Specifically, the antigen-specific type 1 response, especially IgG2-antibody response or IFN-gamma response, of a vaccine against a viral pathogen, especially influenza virus, HCV or HBV, HIV, HPV or JEV, can be improved and at the same time the type 2 response, especially IgG1-antibody response or interleukin 4 (IL 4) response, of said vaccine can be preserved or preferably also increased.

It has been shown previously (WO 02/13857) that naturally occurring, cathelicidin-derived antimicrobial peptides or derivatives thereof have an immune response stimulating activity

and therefore constitute highly effective type 1 inducing adjuvants (Immunizers). Main sources of antimicrobial peptides are granules of neutrophils and epithelial cells lining the respiratory, gastro-intestinal and genitourinary tracts. In general they are found at those anatomical sites most exposed to microbial invasion, are secreted into internal body fluids or stored in cytoplasmic granules of professional phagocytes (neutrophils).

In the WO 02/32451 a type 1 inducing adjuvant (Immunizer) that is able to strongly enhance the immune response to a specific co-administered antigen and therefore constitutes a highly effective adjuvant is disclosed, Peptide A comprising a sequence R_1 -XZXX_nXZX- R_2 . A specifically preferred peptide is KLKLLLLLKLK. Besides naturally occurring antimicrobial peptides, synthetic antimicrobial peptides have been produced and investigated. The synthetic antimicrobial peptide KLKLLLLLKLK-NH₂ was shown to have significant chemotherapeutic activity in *Staphylococcus aureus*-infected mice; human neutrophils were activated to produce the superoxide anion (O_2^-) via cell surface calreticulin. The exact number and position of K and L was found to be critical for the antimicrobial activity of the synthetic peptide (Nakajima, Y. (1997); Cho, J-H. (1999)).

The present invention is especially beneficial if the combined medicament is administered, e.g. subcutaneously, intramuscularly, intradermally or transdermally. However, other application forms, such as parenteral, intravenously, intranasally, oral or topical application, are also suitable for the present invention.

The Influenza antigen may be mixed with the adjuvant (Immunizer) (composition) according to the present invention or otherwise specifically formulated e.g. as liposome, retard formulation, etc..

The vaccines according to the present invention may be administered to an individual in effective amounts known to the skilled man in the art of Influenza vaccination. Optimisation of antigen amount and Immunizer amount can be started from established amounts and using available methods.

The invention will be described in more detail by the following examples and figures, but the invention is of course not limited thereto.

Fig. 1 shows that cationic peptides co-injected with different ODNs synergistically induce strong type 1 humoral responses (IgG2b) against a commercially available Influenza-vaccine;

Fig. 2 shows that KLK/o-d(IC)₁₃ strongly improves the efficacy of a commercially available Influenza vaccine;

Fig. 3 shows that single injection of KLK/o-d(IC)₁₃ synergistically induces strong cellular type 1 and humoral type 1 and 2 responses against a commercially available Influenza vaccine

Fig. 4 shows that single injection of KLK/o-d(IC)₁₃ strongly improves the efficacy of a commercially available Influenza vaccine

Fig. 5 shows that vaccination with ncORF derived peptides from influenza A virus in combination with KLK/o-d(IC)₁₃ induces potent IFN- γ producing T cells and protection against viral challenge

Fig. 6 shows that KLK/O-d(IC)₁₃ induces HCV-peptide specific type 1 cellular immune responses

Fig. 7 shows that cationic peptides co-injected with different ODNs induce a HBsAg-specific cellular type 1 response (IFN- γ production) while the HBsAg-induced type 2 response (IL-4 production) is not affected or decreased.

Fig. 8a shows the results of a single injection of the combination of the cationic antimicrobial peptide KLK with the synthetic oligodeoxynucleotide o-d(IC)₁₃ (ODN1a) and low amounts of a commercially available influenza vaccine (Arippal S1) synergistically induces strong vaccine specific cellular type I immune responses

Fig. 8b shows the results of a single injection of the combination of the cationic antimicrobial peptide KLK with the synthetic oligodeoxynucleotide o-d(IC)13 (ODN1a) and a low amounts of a commercially available influenza vaccine (Agrippal S1) synergistically induces strong mixed type 1/type 2 humoral immune responses

Fig. 9a: shows the results of a single injection of low dose of the combination of the cationic antimicrobial peptide KLK with the synthetic oligodeoxynucleotide o-d(IC)13 (ODN1a) synergistically induces strong cellular type immune responses against a commercially available influenza vaccine (Agrippal S1)

Fig. 9b: shows the results of a single injection of low dose of the combination of the cationic antimicrobial peptide KLK with the synthetic oligodeoxynucleotide o-d(IC)13 (ODN1a) and a low amounts of a commercially available influenza vaccine (Agrippal S1) synergistically induces strong mixed type 1/type 2 humoral immune responses

Fig. 10 shows the results of a single injection of of a commercially available influenza vaccine (Agrippal S1) combined with the cationic antimicrobial peptide KLK and the synthetic oligodeoxynucleotide o-d(IC)13 (ODN1a) compared to other adjuvants

Examples:

Example 1:

Cationic peptides (pR or KLK) co-injected with different oligodeoxynucleotides (ODN) (CpI, ntCpI, o-d(IC)₁₃) synergistically induce strong type 1 humoral responses (IgG2b) against a commercially available Influenza-vaccine (Fluvirin)

Mice C57BL/6 (Harlan/Olac)

Influenza vaccine Fluvirin (Evans vaccine); inactivated Influenza virus surface antigens (haemagglutinin and neuraminidase) purified of strains:

	A/NewCaledonia/20/99 (H1N1)-like strain (15µg haemagglutinin)
	A/Moscow/10/99 (H3N2)-like strain (A/Panama/2007/99 RESVIR-17) (15µg haemagglutinin)
	B/Sichuan/379/99-like strain (15µg haemagglutinin) dose: 1µg total protein/mouse
Al(OH) ₃	Alhydrogel; Biosys, Denmark dose: 1:1 mixture with antigen
pR	Poly-L-Arginine with an average degree of polymerization of 43 arginine residues (determined by MALLS); Sigma Aldrich Inc dose: 100µg/mouse
KLK	KLKLLLLLLKLK-COOH was synthesized by MPS (Multiple Peptide System, USA) dose: 168µg/mouse
oligo-d(IC) ₁₃ (=ODN1a)	ODN 5'ICI CIC ICI CIC ICI CIC ICI CIC IC3' was synthesized by Purimex Nucleic Acids Technology, Göttingen dose : 5nmol/mouse
I-ODN 2	thiophosphate substituted ODNs containing deoxyinosine: tcc atg aci ttc ctg atg ct, were synthesized by Purimex Nucleic Acids Technology, Göttingen dose: 5nmol/mouse
I-ODN 2b	ODNs containing deoxyinosine: tcc atg aci ttc ctg atg ct, were synthesized by Purimex Nucleic Acids Technology, Göttingen dose: 5nmol/mouse
formulation	5mM Tris/270mM Sorbitol, pH 7

experimental group (12 mice/group):

1. : naïve
2. : Flu vaccine
3. : Flu vaccine + pR
4. : Flu vaccine + KLK
5. : Flu vaccine + Al(OH)₃
6. : Flu vaccine + o-d(IC)₁₃
7. : Flu vaccine + I-ODN 2
8. : Flu vaccine + I-ODN 2b
9. : Flu vaccine + pR + I-ODN 2
- 10.: Flu vaccine + KLK + o-d(IC)₁₃
- 11.: Flu vaccine + KLK + I-ODN 2
- 12.: Flu vaccine + KLK + I-ODN 2b

On days 0, 28 and 56 C57BL/6 mice were injected s.c. into both hind footpads with a total volume of 100µl/mouse (50µl/footpad) containing the above listed compounds. Serum was collected at days 26, 54 and 82 and analyzed for Influenza vaccine-specific IgG1 and IgG2b antibodies by ELISA. Titers correspond to that dilution of serum resulting in half maximal OD_{405nm}.

Figure 1 indicates that the combined injection of cationic peptides (pR or KLK) and different ODNs (I-ODN 2, I-ODN 2b, or o-d(IC)₁₃) induces very potent antigen (Influenza vaccine)-specific humoral type 1 responses (IgG2b) in a synergistic way. Upon injection of Influenza vaccine alone or in combination with Al(OH)₃, cationic peptides (pR, KLK) only or different ODNs (except I-ODN 2) only, no specific IgG2b response is detectable. Booster vaccinations strongly increase the observed response.

Co-injection of Influenza vaccine with Al(OH)₃, KLK or combinations pR/I-ODN 2, KLK/I-ODN 2, KLK/I-ODN 2b or KLK/o-d(IC)₁₃ induces the production of Influenza vaccine-specific IgG1 (type 2 response).

Example 2:

The combination KLK/o-d(IC)₁₃ strongly improves the efficacy of a commercially available Influenza-vaccine (Fluvirin)

Mice	BALB/c (Harlan/Olac)
Influenza vaccine	Fluvirin (Evans vaccine); inactivated Influenza virus surface antigens (haemagglutinin and neuraminidase) purified of strains: A/NewCaledonia/20/99 (H1N1)-like strain (15µg haemagglutinin) A/Moscow/10/99 (H3N2)-like strain (A/Panama/2007/99 RESVIR-17) (15µg haemagglutinin) B/Sichuan/379/99-like strain (15µg haemagglutinin) dose: 1µg total protein/mouse (= low dose / literature: 10µg/mouse)
Al(OH) ₃	Alhydrogel; Biosys, Denmark dose: 1:1 mixture with antigen
KLK	KLKLLLLLLKLK-COOH was synthesized by MPS (Multiple Peptide System, USA) dose: 168µg/mouse
oligo-d(IC) ₁₃ (=ODN1a)	ODN 5'ICI CIC ICI CIC ICI CIC ICI CIC IC3' was synthesized by Purimex Nucleic Acids Technology, Göttingen dose : 5nmol/mouse
formulation	5mM Tris/270mM Sorbitol, pH 7

experimental group (12 mice/group):

1. naïve
2. Flu vaccine
3. Flu vaccine + Al(OH)₃
4. Flu vaccine + KLK + o-d(IC)₁₃

On days 0, 28 and 56 BALB/c mice were injected s.c. into both hind footpads with a total volume of 100µl/mouse (50µl/footpad) containing the above listed compounds. Serum was collected at

days 26, 54 and 82 and analyzed for neutralizing anti-haemagglutinin antibodies by using a standard haemagglutination inhibition assay. Briefly, the presence of haemagglutinin on the virus surface induces haemagglutination of erythrocytes, which can be inhibited by neutralizing anti-haemagglutinin antibodies. Titers of antibodies against haemagglutinin of the different viral strains (A1 = A/NewCaledonia/20/99 (H1N1)-like strain; A2 = A/Panama/2007/99 RESVIR-17; B = B/Sichuan/379/99-like strain) were determined. Titer of serum corresponds to end point dilution showing inhibition.

In contrast to injection of Influenza vaccine alone or in combination with Al(OH)₃, the co-injection of Influenza vaccine plus KLK and o-d(IC)₁₃ induces high levels of neutralizing antibodies against all three tested haemagglutinins (Fig. 2). Since effectiveness of an Influenza vaccine has been shown to correlate with serum titers of anti-haemagglutinin antibodies the obtained results indicate a high potential of KLK/o-d(IC)₁₃ for the induction of protection against Influenza.

Example 3:

Single injection of the combination of the cationic antimicrobial peptide KLK and the synthetic oligodeoxynucleotide o-d(IC)₁₃ synergistically induces strong cellular type 1 and humoral type 1 / type 2 immune responses against a commercially available influenza vaccine (Agrippal S1)

Materials

Mice

BALB/c (Harlan-Winkelmann,
Germany)

Influenza vaccines

Agrippal S1 (Chiron SpA, Italy; season 2002/2003); inactivated purified influenza virus antigens (hemagglutinin and neuraminidase) from strains:
A/New Caledonia/20/99 (H1N1) - like strain
(A/New Caledonia/20/99 IVR-116)
A/Moscow/10/99 (H3N2) - like strain
(A/Panama/2007/99 RESVIR 17)

B/Hong Kong/330/2001 - like strain
(B/Shangdong/7/97)

Total antigen content: 45µg (15µg for
each antigen); Lot #4307; expiry date
05/2003

Dose: 1µg total protein/mouse

Fluad (Chiron SpA, Italy; season
2002/2003); inactivated purified
influenza virus antigens (hemagglutinin
and neuraminidase) from strains:

A/New Caledonia/20/99 (H1N1) - like
strain

(A/New Caledonia/20/99 IVR-116)

A/Moscow/10/99 (H3N2) - like strain

(A/Panama/2007/99 RESVIR 17)

(B/Shangdong/7/97)

Total antigen content: 45µg (15µg for
each antigen); Addition of MF59C.1 as
adjuvant

Lot #3403; expiry date 05/2003

Dose: 1µg total protein/mouse

o-d(IC)₁₃ (=ODN1a)

ODN 5' ICI CIC ICI CIC ICI CIC ICI CIC
IC3'

synthesized by Purimex Nucleic Acids
Technology, Göttingen

Dose: 0.4nmol/mouse

KLK

KLKLLLLLKLK-COOH

synthesized by MPS (Multiple Peptide
System, USA)

Dose: 10nmol/mouse

Formulation

10mM Tris/135mM NaCl ; pH ~7

Experimental setup (10 mice/group)

1. naïve

2. Agrippal S1
3. Fluad
4. Agrippal S1 + KLK + o-d(IC)₁₃

On day 0 BALB/c mice were injected intramuscularly into both hind femoral muscles with a total amount of 100µl vaccine/mouse (50µl/muscle) containing the above listed compounds. On day 21, serum was collected and analyzed for influenza vaccine-specific IgG1 and IgG2a antibodies by ELISA. Titers correspond to the dilution of serum resulting in half maximal OD_{405nm}. Furthermore, spleens of each experimental group were pooled and single cell suspensions were prepared. An aliquot of splenocytes was separated into CD4⁺ T cells by magnetic sorting (CD4 MACS sort, Miltenyi). Either unseparated splenocytes or separated CD4⁺ T cells in combination with irradiated antigen-presenting cells (APC; derived from naïve mice) were stimulated in 96-well ELISPOT plates in order to enumerate the number of Agrippal S1 antigen-specific cytokine-producing cells for each experimental group. The production of following cytokines was analyzed:

IFN-γ (as an indicator for a cellular type 1 response),

IL-4 and IL-5 (as indicators for a cellular type 2 response)

Results (Fig. 3a)

Injection of low amounts of influenza vaccines Agrippal S1 (non-adjuvanted) and Fluad (MF59 adjuvanted) alone are not able to induce vaccine (Agrippal S1)-specific IFN-γ by CD4⁺ T cells, whereas upon injection of Agrippal S1 in combination with KLK/o-d(IC)₁₃ a strong vaccine (Agrippal S1)-specific IFN-γ production by CD4⁺ T cells is observed. Compared to naïve mice, Agrippal S1 alone only slightly induces the production of IL-4 by CD4⁺ T cells and the addition of KLK/o-d(IC)₁₃ to the vaccine shows no further increase. However, Fluad is a potent inducer of IL-4 production by CD4⁺ T cells and IL-5 production by unseparated splenocytes. IL-5 is only detectable at very low levels upon injection of Agrippal S1 alone, but not in combination with KLK/o-d(IC)₁₃. Upon restimulation of unseparated splenocytes similar results are obtained

Results (Fig. 3b)

Fig. 3b shows that the injection of the adjuvanted influenza vaccine Fluad alone induces a strong vaccine (Agrippal S1)-specific humoral type 2 response (IgG1), but only a weak type 1 response (IgG2a). However, the combined injection of the non-adjuvanted influenza vaccine with KLK/o-d(IC)₁₃ induces very potent vaccine (Agrippal S1)-specific IgG2a (humoral type 1 immune response) and higher levels of IgG1 than Agrippal S1 alone. Since protection against influenza is correlated with the presence of vaccine antigen-specific IgG2a antibodies, the obtained results indicate a high potential of KLK/o-d(IC)₁₃ as a potent adjuvant for influenza vaccines.

Example 4:

The combination KLK/o-d(IC)₁₃ strongly improves the efficacy of a commercially available influenza vaccine (Agrippal S1) upon single injection

Materials

Mice

BALB/c (Harlan-Winkelmann, Germany)

Influenza vaccines

Agrippal S1 (Chiron SpA, Italy; season 2002/2003); inactivated influenza virus antigens (hemagglutinin and neuraminidase) purified of strains:

A/New Caledonia/20/99 (H1N1) - like strain

(A/New Caledonia/20/99 IVR-116)

A/Moscow/10/99 (H3N2) - like strain

(A/Panama/2007/99 RESVIR 17)

B/Hong Kong/330/2001 - like strain

(B/Shangdong/7/97)

Total antigen content: 45µg (15µg for each antigen); Lot #4307; expiry date 05/2003

Dose: 1µg total protein/mouse

Fluad (Chiron SpA, Italy; season 2002/2003); inactivated influenza virus

antigens (hemagglutinin and neuraminidase) purified of strains:

A/New Caledonia/20/99 (H1N1) - like strain

(A/New Caledonia/20/99 IVR-116)

A/Moscow/10/99 (H3N2) - like strain

(A/Panama/2007/99 RESVIR 17)

(B/Shangdong/7/97)

Total antigen content: 45µg (15µg for each antigen); Addition of MF59C.1 as adjuvant

Lot #3403; expiry date 05/2003

Dose: 1µg total protein/mouse

o-d(IC)₁₃ (=ODN1a)

ODN 5'ICI CIC ICI CIC ICI CIC ICI CIC IC3'

was synthesized by Purimex Nucleic Acids Technology, Göttingen

Dose: 0.5nmol/mouse

KLK

KLKLLLLLLKLK-COOH

was synthesized by MPS (Multiple Peptide System, USA)

Dose: 10nmol/mouse

Formulation

10mM Tris/270mM Sorbit; pH ~ 7

Experimental setup (10 mice/group)

1. naïve
2. Agrippal S1
3. Fluad
4. Agrippal S1 + KLK + o-d(IC)₁₃

On day 0 BALB/c mice were injected intramuscularly into both hind femoral muscles with a total amount of 100µl vaccine/mouse (50µl/muscle) containing the above listed compounds. On day 21, serum was collected and analyzed for neutralizing anti-

hemagglutinin antibodies by the use of a standard hemagglutinin inhibition (HI) assay for human sera. Titers of antibodies against hemagglutinin derived from different viral strains of both influenza vaccines Agrippal S1 and Fluad (see Materials) were determined.

Results (Fig. 4)

In contrast to injection of Agrippal S1 alone, the co-injection of the influenza vaccine with low amounts of KLK/o-d(IC)13 induces strongly increased levels of neutralizing antibodies against the two influenza A strains tested (A/New Caledonia/20/99; A/Panama/2007/99). However, immunisation with Fluad induces the same levels of neutralizing antibodies as co-injection of Agrippal S1 with low amounts of KLK/o-d(IC)13. Since effectiveness of an influenza vaccine has been shown to correlate with serum titers of anti-hemagglutinin antibodies the present results indicate a high potential of KLK/ o-d(IC)13 as an adjuvant for the induction of protection against influenza.

Example 5:

Vaccination of mice with ncORF derived peptides from influenza A virus in combination with KLK / o-d(IC)₁₃. Specific T-cell response is measured 7 days after vaccination, and animals are subsequently challenged with a lethal dose of mouse adapted influenza A virus (x31). Survival is monitored for 15 days.

Materials

Mice	C57Bl/6 (Harlan-Winkelmann, Germany)
Peptides	p82 (GLCTLVAML) Control peptide derived from EBV ; HLA-A*0201; AA start 280 p1574 (IASNENMETM) Control peptide derived from Influenza nucleoprotein, AA start 365 p1569 (TMLYNKMEF) Flu ncORF derived peptide from segment

1, frame 1, ORF 1, AA start 569

p1600 (SSIAAQDAL)

Flu ncORF derived peptide from segment
3, frame 6, ORF 2, AA start 83

P1664 (VTILNLALL)

Flu ncORF derived peptide from segment
4, frame 5, ORF 6, AA start 9

Dose: 100µg/peptide/mouse

o-d(IC)₁₃
(=ODN1a)

ODN 5' ICI CIC ICI CIC ICI CIC ICI CIC
IC3'

was synthesized by Purimex Nucleic Acids
Technology, Göttingen

Dose: 5nmol/mouse

KLK

KLKLLLLLLKLK-COOH

was synthesized by MPS (Multiple Peptide
System, USA)

Dose: 127nmol/mouse

Formulation

270mM Sorbit/10mM Hepes

**Influenza A
virus**

x31, mouse adapted influenza A virus,
rec. virus derived from A/Pr/8/34 (seg
1, 2, 3, 5, 7, 8) and A/Aichi/2/68 (seg
4, 6)

Experimental setup (15 mice/group)

1. p1574 + KLK + o-d(IC)₁₃
2. p1569 + KLK + o-d(IC)₁₃
3. p1600 + KLK + o-d(IC)₁₃
4. p1664 + KLK + o-d(IC)₁₃
5. p1600 + p1569 + KLK + o-d(IC)₁₃

On day 0 mice were injected s.c into both hind footpads with a
total amount of 100µl vaccine/mouse (50µl/foot) containing the
above listed compounds. On day 7, unseparated splenocytes from 5

mice were stimulated in 96-well ELISpot plates in order to enumerate the number of peptide-specific IFN- γ producing cells for each experimental group.

Remaining 10 mice were challenged with mouse adapted x31 influenza A virus (5×10^5 pfu). Survival was monitored for 15 days.

Results ELISpot (Fig. 5a)

Spleen cells of groups 1 and 3 (peptides p1574 and p1600) do not show any specific spots after restimulation with the respective peptides. Groups 2 and 4 (p1569 and p1664) specifically release IFN- γ after restimulation. Group 5 was vaccinated with two individual peptides (not as a mix, p1600 and p1569). Upon restimulation with either the mix of both peptides or p1569, specific cytokine release is detected. In contrast, upon restimulation with p1600 alone, no IFN- γ spots are detectable. This is consistent with group 3 (p1600 alone).

Results challenge (Fig. 5b)

Fig. 5b shows the survival rate of challenged mice with a lethal dose of mice adapted influenza A virus x31. Group 1 (p1574, reported protective epitope for H2-Db) protects 30% of all challenged mice. Peptide p1569 does not at all provide protection (0%). In contrast, peptides p1600 and p1664 do protect 50% and 62% of challenged animals, respectively. When animals are vaccinated with two different peptides (group 5, peptides p1600 and 1569) up to 70% of animals are protected.

Example 6:

Potent HCV-specific type 1 cellular responses are induced by the combined injection of five different HCV-derived peptides, the antimicrobial peptide KLK and the synthetic oligodeoxynucleotide o-d(IC)₁₃.

Mice HLA-A*0201 transgenic mice (HHD.1)

Peptides	The peptides p83, p84, p87, p89, p1426 were used for vaccination.
----------	---

p83: HCV-derived peptide,

(KFPGGGQIVGGVYLLPRRGPRL)

p84: HCV-derived peptide,
(GYKVLVLNPSVAAT)

p87: HCV-derived peptide,
(DLMGYIPAV)

p89: HCV-derived peptide,
(CINGVCWTV)

p1426: HCV-derived peptide,
(HMWNFISGIQYLAGLSTLPGNPA)

(p1274 used for restimulation as irrelevant
peptide
(YMDGTMSQV; HLA-A*0201 restricted)

All peptides were synthesized by standard
solid phase F-moc synthesis, HPLC purified
and analyzed by mass spectroscopy for
purity.

dose: 20µg per peptide/mouse

KLK

KLKLLLLLLKLK-COOH was synthesized by MPS
(Multiple Peptide System, USA)

dose: 10nmol/mouse

oligo-d(IC)₁₃ (= ODN1a) ODN 5'ICI CIC ICI CIC ICI CIC ICI CIC IC3'
was synthesized by Purimex Nucleic Acids
Technology, Göttingen

dose: 0.4nmol/mouse

Formulation

10mM Tris/135mM NaCl; pH ~7

Experimental setup (5 mice/group):

1. HCV peptides
2. HCV peptides + KLK + o-d(IC)₁₃

On days 0, 14 and 28 HHD.1 mice were injected s.c. into both hind footpads with a total volume of 100µl/mouse (50µl/footpad) containing the above listed compounds. At day 35 (7 days after last vaccination) CD4⁺ as well as CD8⁺ T cells were isolated by magnetic separation (MACS, Miltenyi) from single cell suspensions of spleen cells. T cells were incubated with medium (background control) or were restimulated with irradiated spleen cells from naïve HHD.1 mice as APC in the presence of either the different peptides used for vaccination or the irrelevant peptide p1274. After overnight incubation, the IFN-γ production was analyzed using an ELISpot assay.

Figure 6 shows that upon co-injection of the five HCV-derived peptides with KLK/o-d(IC)₁₃ high amounts of IFN-γ produced by CD4⁺ T cells against p84, p87, p89, p1426 were induced. Furthermore, a strong IFN-γ production by CD8⁺ T cells against p87, p89 was detectable.

Example 7:

Cationic peptides (pR or KLK) co-injected with different oligodeoxynucleotides (ODN) (CpI, ntCpI, o-d(IC)₁₃) synergistically induce strong type 1 cellular responses (IFN-γ) against Hepatitis B surface Antigen

Mice	C57BL/6 (Harlan-Winkelmann, Germany); low responder mice for HbsAg-specific immune responses
Antigen	Hepatitis B surface antigen (HBsAg) dose: 5µg / mouse
Al(OH) ₃	Alhydrogel; Biosys, Denmark dose: 1:1 mixture with antigen

pR

Poly-L-Arginine with an average degree of polymerization of 43 arginine residues (determined by MALLS); Sigma Aldrich Inc

dose: 100µg / mouse

KLK

KLKLLLLLLKLK-COOH was synthesized by MPS (Multiple Peptide System, USA)

dose: 168µg / mouse

I-ODN 2

(= CpI 2) thiophosphate substituted ODNs containing deoxyinosines: 5'tcc atg aci ttc ctg atg ct 3' were synthesized by Purimex Nucleic Acids Technology, Göttingen

dose: 5nmol / mouse

I-ODN 2b

(= CpI 2b) ODNs containing deoxyinosines: 5'tcc atg aci ttc ctg atg ct 3' were synthesized by Purimex Nucleic Acids Technology, Göttingen

dose: 5nmol / mouse

o-d(IC)₁₃ (=ODN1a)

ODN 5'ICI CIC ICI CIC ICI CIC ICI CIC IC3' was synthesized by Purimex Nucleic Acids Technology, Göttingen

dose: 5nmol / mouse

Formulation

5mM Tris/270mM Sorbitol, pH7

Experimental setup (5 mice/group/timepoint):

- | | | | |
|----------|-------------------------|------------|-------------------------|
| 1. HBsAg | | | |
| 2. HBsAg | + Alum | | |
| 3. HBsAg | + I-ODN 2 | | |
| 4. HBsAg | + I-ODN 2b | | |
| 5. HBsAg | + o-d(IC) ₁₃ | | |
| 6. HBsAg | + pR | | |
| 7. HBsAg | + KLK | | |
| 8. HBsAg | + pR | + I-ODN 2 | |
| 9. HBsAg | + pR | + I-ODN 2b | |
| 10. | HBsAg | + pR | + o-d(IC) ₁₃ |
| 11. | HBsAg | + KLK | + I-ODN 2 |
| 12. | HBsAg | + KLK | + I-ODN 2b |
| 13. | HBsAg | + KLK | + o-d(IC) ₁₃ |

On day 0 and day 56 mice were injected subcutaneously into the right flank with a total volume of 100µl/mouse containing the above mentioned compounds. The analysis of the immune response was performed at day 7, day 21 and day 50 after first and second injection, respectively. Spleen cells of five mice per group per time point were restimulated ex vivo with 10µg/ml HBsAg and ELISpot assays were performed in order to analyse the HBsAg-specific IFN-γ (type 1 immune response) as well as IL-4 (type 2 immune response) production.

Results (Fig. 7)

HBsAg injected alone or in combination with Alum induces no or only very low levels of IFN-γ, whereas upon injection of HBsAg combined with pR/ODN or KLK/ODN an HBsAg-specific IFN-γ production is induced which can be further increased by booster vaccination. Slightly increased IL-4 production compared to injection of HBsAg alone is observable upon co-injection of Alum, pR and KLK after boost, as well upon co-injection of KLK/ODN combinations.

Example 8:

Single injection of the combination of the cationic antimicrobial peptide .KLK with the synthetic

oligodeoxynucleotide o-d(IC)₁₃ (ODN1a) and low amounts of a commercially available influenza vaccine (Agrippal S1) synergistically induces strong vaccine specific cellular type I immune responses

Materials

Mice

BALB/c (Harlan-Winkelmann, Germany).

Influenza vaccine

Agrippal S1 (Chiron SpA, Italy; vaccination season 2003/2004); lot 035105, expiry date 06/2004

Inactivated influenza virus antigens (haemagglutinin and neuraminidase) purified of strains:

A/New Caledonia/20/99 (H1N1) - like strain (A/New Caledonia/20/99 IVR-116)

A/Moscow/10/99 (H3N2) - like strain (A/Panama/2007/99 RESVIR 17)

B/Hong Kong/330/2001 - like strain (B/Shangdong/7/97)

Total antigen content: 45µg

haemagglutinin (15µg for each viral strain)

Dose: 9µg total protein / mouse (used directly from pre-filled syringe) 0.1µg total protein / mouse

o-d(IC)₁₃ (=ODN1a)

phosphodiester substituted ODN containing 13 deoxy (-inosine, -cytosine) motifs (ICI CIC ICI CIC ICI CIC ICI CIC IC); synthesized by Transgenomics; Part number: I02A03001N

Dose: 4nmol / mouse
1.4nmol / mouse
0.4nmol / mouse

KLK

synthetic cationic poly-amino acid containing lysine and leucine

(KLKLLLLLLKLK-COOH); synthesized by Bachem AG

Lot: 0562101;

Dose: 100nmol / mouse

35nmol / mouse

10nmol / mouse

Formulation by the Pharmaceutical Development Department at Intercell; 10mM Tris / 135mM NaCl pH 7.5

Experimental Setup (10 mice / group)

1. naïve
2. 9µg Agrippal S1
3. 0.1µg Agrippal S1
4. 0.1µg Agrippal S1 + 100nmol KLK + 4nmol ODN1a
5. 0.1µg Agrippal S1 + 35nmol KLK + 1.4nmol ODN1a
6. 0.1µg Agrippal S1 + 10nmol KLK + 0.4nmol ODN1a

On day 0, BALB/c mice were injected intramuscularly into both femoral muscles with a total amount of 100µl vaccine / mouse (50µl / muscle) containing the above listed compounds. On day 21, serum was collected and analyzed for influenza vaccine-specific IgG1 and IgG2a antibodies by ELISA. Titers correspond to the dilution of serum resulting in half maximal OD_{405nm}. Furthermore, spleens of each experimental group were pooled and single cell suspensions were prepared. An aliquot of splenocytes was separated into CD4⁺ and CD8⁺ T cells by magnetic sorting (CD4 and CD8 MACS sort, Miltenyi). Either unseparated splenocytes or separated CD4⁺ and CD8⁺ T cells in combination with irradiated antigen-presenting cells (APC; derived from naïve mice) were stimulated in 96-well ELISPOT plates in order to enumerate the number of Agrippal S1 antigen-specific cytokine-producing cells for each experimental group. The production of following cytokines was analyzed:

IFN-γ (as an indicator for a cellular type 1 response),

IL-4 and IL-5 (as indicators for a cellular type 2 response)

Results (Fig. 8a)

Vaccination of mice with 9µg and 0.1µg Agrippal S1 alone was not able to induce vaccine-specific IFN-γ by CD4⁺ splenocytes, whereas upon injection of Agrippal S1 in combination with different concentrations of KLK/o-d(IC)₁₃ a strong IFN-γ production by CD4⁺ splenocytes was observed. However, CD8⁺ splenocytes were not able to induce IFN-γ. Compared to naïve mice, Agrippal S1 alone only slightly induced the production of IL-4 by unseparated and CD4⁺ splenocytes and the addition of KLK/o-d(IC)₁₃ to the vaccine showed no further increase. However, Agrippal S1 induced IL-5 was totally abolished upon co-injection of KLK/o-d(IC)₁₃. Upon restimulation of unseparated splenocytes similar results are obtained.

Results (Fig. 8b)

As illustrated in Fig. 8b, the combined injection of low amounts of the non-adjuvanted influenza vaccine with different concentrations of KLK/o-d(IC)₁₃ induced very potent vaccine (Agrippal S1)-specific IgG2a (humoral type 1 immune response) and higher levels of IgG1 than low amounts of Agrippal S1 alone. However, the high dose of Agrippal S1 showed the highest titers of vaccine-specific IgG1 antibodies. Since protection against influenza is correlated with the presence of vaccine antigen-specific IgG2a antibodies, the obtained results indicate a high potential of KLK/o-d(IC)₁₃ as a potent adjuvant for influenza vaccines.

Example 9:

Single injection of low dose of the combination of the cationic antimicrobial peptide KLK with the synthetic oligodeoxynucleotide o-d(IC)₁₃ (ODN1a) synergistically induces strong vaccine specific cellular type I immune responses against a commercially available influenza vaccine (Agrippal S1)

Materials

Mice	BALB/c (Harlan-Winkelmann, Germany)
Influenza vaccine	Agrippal S1 (Chiron SpA, Italy; vaccination season 2003/2004); lot 035105, expiry date 06/2004

Inactivated influenza virus antigens
(haemagglutinin and neuraminidase)
purified of strains:

A/New Caledonia/20/99 (H1N1) - like
strain (A/New Caledonia/20/99 IVR-116)

A/Moscow/10/99 (H3N2) - like strain
(A/Panama/2007/99 RESVIR 17)

B/Hong Kong/330/2001 - like strain
(B/Shangdong/7/97)

Total antigen content: 45µg
haemagglutinin (15µg for each viral
strain)

Dose: 9µg total protein / mouse (used
directly from pre-filled syringe)
1µg total protein / mouse

o-d(IC)₁₃ (=ODN1a) phosphodiester substituted ODN containing
13 deoxy (-inosine, -cytosine) motifs
(ICI CIC ICI CIC ICI CIC ICI CIC IC);
synthesized by Transgenomics; Part
number: I02A03001N

Dose: 0.4nmol / mouse
0.2nmol / mouse
0.04nmol / mouse

KLK synthetic cationic poly-amino acid
containing lysine and leucine
(KLKLLLLLLKLK-COOH); synthesized by Bachem
AG

Lot: 0562101;

Dose: 10nmol / mouse
5nmol / mouse
1nmol / mouse

Formulation by the Pharmaceutical Development
Department at Intercell; 10mM Tris /
135mM NaCl pH 7.5

Experimental Setup (5 mice / group)

1. naïve
2. 9µg Agrippal S1
3. 1µg Agrippal S1
4. 1µg Agrippal S1 + 10nmol KLK + 0.4nmol ODN1a
5. 1µg Agrippal S1 + 5nmol KLK + 0.2nmol ODN1a
6. 1µg Agrippal S1 + 1nmol KLK + 0.04nmol ODN1a

On day 0, BALB/c mice were injected intramuscularly into both femoral muscles with a total amount of 100µl vaccine / mouse (50µl / muscle) containing the above listed compounds. On day 21, serum was collected and analyzed for influenza vaccine-specific IgG1 and IgG2a antibodies by ELISA. Titers correspond to the dilution of serum resulting in half maximal OD405_{nm}. Furthermore, spleens of each experimental group were pooled and single cell suspensions were prepared. Splenocytes were stimulated in 96-well ELISPOT plates in order to enumerate the number of Agrippal S1 antigen-specific cytokine-producing cells for each experimental group. The production of following cytokines was analyzed:

IFN-γ (as an indicator for a cellular type 1 response),
IL-4 and IL-5 (as indicators for a cellular type 2 response)

Results (Fig. 9a)

Vaccination of mice with 9µg and 1µg Agrippal S1 alone was again (see also Fig. 8a) not able to induce vaccine-specific IFN-γ by murine splenocytes, whereas upon injection of Agrippal S1 in combination with different low concentrations of KLK/o-d(IC)₁₃ a strong IFN-γ production was observed. Compared to naïve mice, Agrippal S1 alone only slightly induced the production of IL-4 by murine splenocytes and the addition of KLK/o-d(IC)₁₃ to the vaccine had no effect. Agrippal S1 induced IL-5 was abolished upon co-injection of KLK/o-d(IC)₁₃ in a concentration dependent manner. Even at a very low dosage, the combination of KLK/o-d(IC)₁₃ induces a strong cellular type I immune responses.

Results (Fig. 9b)

The combined injection of low amounts of the non-adjuvanted influenza vaccine with different concentrations of KLK/o-d(IC)₁₃ induced very potent vaccine (Agrippal S1)-specific IgG2a

(humoral type 1 immune response) and levels of **IgG1** roughly comparable to low amounts of Agrippal S1 alone. However, the high dose of Agrippal S1 alone showed slightly increased titers of vaccine-specific IgG1 antibodies and comparable levels of IgG2a antibodies compared to co-injection of Agrippal S1 and the lowest concentration of KLK/o-d(IC)₁₃. Since protection against influenza is correlated with the presence of vaccine antigen-specific IgG2a antibodies, the obtained results indicate a high potential of KLK/o-d(IC)₁₃, even at a very low dosage, as a potent adjuvant for influenza vaccines.

Example 10

Single injection of a commercially available influenza vaccine (Agrippal S1) combined with the cationic antimicrobial peptide KLK and the synthetic oligodeoxynucleotide o-d(IC)₁₃ (ODN1a) in comparison to other adjuvants

Materials

Mice

BALB/c (Harlan-Winkelmann, Germany)

Influenza vaccines

Agrippal S1 (Chiron SpA, Italy; vaccination season 2003/2004); lot 035105, expiry date 06/2004 and

Fluad (Chiron SpA, Italy; vaccination season 2003/2004); lot 4003, expiry date 05/2004; Addition of MF59C.1 as adjuvant

Both vaccines contain inactivated influenza virus antigens (haemagglutinin and neuraminidase) purified of strains:

A/New Caledonia/20/99 (H1N1) - like strain (A/New Caledonia/20/99 IVR-116)

A/Moscow/10/99 (H3N2) - like strain (A/Panama/2007/99 RESVIR 17)

B/Hong Kong/330/2001 - like strain (B/Shangdong/7/97)

Total antigen content: 45µg

haemagglutinin (15µg for each viral strain)

Dose: 9µg total protein / mouse (used
directly from pre-filled syringe)
1µg total protein / mouse

o-d(IC)₁₃ (=ODN1a) phosphodiester substituted ODN containing
13 deoxy (-inosine, -cytosine) motifs
(ICI CIC ICI CIC ICI CIC ICI CIC IC);
synthesized by Transgenomics; Part
number: I02A03001N
Dose: 0.4nmol / mouse

KLK synthetic cationic poly-amino acid
containing lysine and leucine
(KLKLLLLLLKLK-COOH); synthesized by Bachem
AG
Lot: 0562101;
Dose: 100nmol / mouse
10nmol / mouse

CpG1668 Thiophosphate-substituted ODN containing
CpG motifs (5'-tcc atg acg ttc ctg atg
ct-3'); synthesized by Purimex Nucleic
Acids Technology, Göttingen; lot. 020614
Dose: 5nmol / mouse

Alum Al (OH)₃ [15g/l]; provided by Chiron
Behring, Germany
Dose: 1:1 mixture with antigen

pR Poly-L-Arginine with an average degree of
polymerization of 43 arginine residues
(determined by MALLS); provided by Sigma;
lot 50K7280;
Dose: 100µg / mouse

IFA Incomplete Freud's Adjuvant; provided by
Difco Lab.; lot. 2158430
Dose: 1:1 mixture with antigen

CFA Complete Freud's Adjuvant; provided by
Difco Lab.; lot. 3126639
Dose: 1:1 mixture with antigen

Formulation by the Pharmaceutical Development
Department at Intercell; 10mM Tris /
135mM NaCl pH 7.5

Experimental Setup (5 mice / group)

1. naïve
2. 9µg Fluad
3. 9µg Agrippal S1
4. 1µg Agrippal S1
5. 1µg Agrippal S1 + 10nmol KLK + 0.4nmol ODN1a
6. 1µg Agrippal S1 + 5nmol CpG1668
7. 1µg Agrippal S1 + 100µg pR43
8. 1µg Agrippal S1 + 100nmol KLK
9. 1µg Agrippal S1 + Alum
10. 1µg Agrippal S1 + CFA
11. 1µg Agrippal S1 + IFA

On day 0, BALB/c mice were injected intramuscularly into both femoral muscles with a total amount of 100µl vaccine / mouse (50µl / muscle) containing the above listed compounds. On day 21, spleens of each experimental group were pooled and single cell suspensions were prepared. Splenocytes were stimulated in 96-well ELIspot plates in order to enumerate the number of Agrippal S1 antigen-specific cytokine-producing cells for each experimental group. The production of following cytokines was analyzed:

IFN-γ (as an indicator for a cellular type 1 response),
IL-4 and IL-5 (as indicators for a cellular type 2 response)

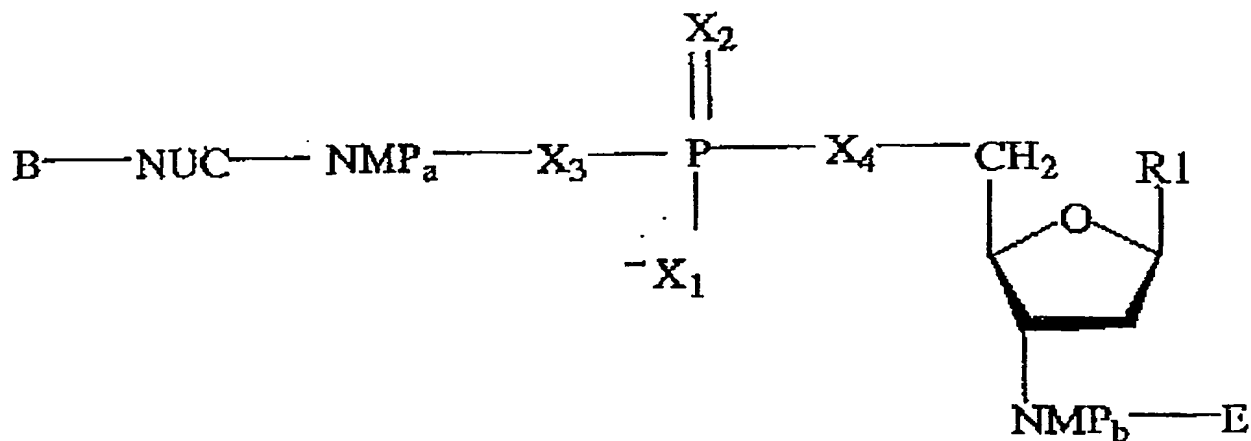
Results (Fig. 10)

The combined injection of low amounts of the non-adjuvanted influenza vaccine with low doses KLK/o-d(IC)₁₃ induced higher levels vaccine (Agrippal S1)-specific IFN-γ by murine splenocytes compared to immunisation with Agrippal S1 and Fluad alone. Comparable amounts of IFN-γ were only achieved upon vaccination

with CFA, all other experimental groups showed roughly no (pR43, Alum, IFA) or a slightly increased (CpG1668, KLK) IFN- γ production by murine splenocytes compared to Agrippal S1 alone. In comparison to naïve mice, only Flud markedly induced the production of **IL-4** by murine splenocytes whereas all other experimental groups nearly showed the same low levels of IL-4. Vaccine antigen specific **IL-5** was detectable at very high levels upon vaccination of mice with Flud and at moderate levels upon injection of Agrippal S1 alone or in combination with KLK. Co-injection of Agrippal S1 with pR, Alum or IFA only slightly increased levels of IL-5, whereas all other groups showed a reduction in IL-5 production.

Claims:

1. Vaccine for preventing viral infections comprising
- an antigen,
 - a peptide comprising a sequence $R_1\text{-XZXXZ}_N\text{XZX-R}_2$, whereby N is a whole number between 3 and 7, preferably 5, X is a positively charged natural and/or non-natural amino acid residue, Z is an amino acid residue selected from the group consisting of L, V, I, F and/or W, and R_1 and R_2 are selected independantly one from the other from the group consisting of -H, -NH₂, -COCH₃, -COH, a peptide with up to 20 amino acid residues or a peptide reactive group or a peptide linker with or without a peptide; X-R₂ may be an amide, ester or thioester of the C-terminal amino acid residue of the peptide ("Peptide A"), and
 - an immunostimulatory oligodeoxynucleic acid molecule (ODN) having the structure according to the formula (I)



wherein

R1 is selected from hypoxanthine and uracile,

any X is O or S,

any NMP is a 2' deoxynucleoside monophosphate or monothiophosphate, selected from the group consisting of deoxyadenosine-, deoxyguanosine-, deoxyinosine-, deoxycytosine-, deoxyuridine-, deoxythymidine-, 2-methyl-deoxyinosine-, 5-methyl-deoxycytosine-, deoxypseudouridine-, deoxyribosepurine-, 2-amino-deoxyribosepurine-, 6-S-deoxyguanine-, 2-dimethyl-deoxyguanosine- or N-isopentenyl-deoxyadenosine-monophosphate or -monothiophosphate,

NUC is a 2' deoxynucleoside, selected from the group consisting of deoxyadenosine-, deoxyguanosine-, deoxyinosine-, deoxycytosine-, deoxyinosine-, deoxythymidine-, 2-methyl-deoxyuridine-, 5-methyl-deoxycytosine-, deoxypseudouridine-, deoxyribosepurine-, 2-amino-deoxyribosepurine-, 6-S-deoxyguanine-, 2-dimethyl-deoxyguanosine- or N-isopentenyl-deoxyadenosine,

a and b are integers from 0 to 100 with the proviso that a + b is between 4 and 150, and

B and E are common groups for 5' or 3' ends of nucleic acid molecules ("I-/U-ODN").

2. Vaccine according to claim 1, characterised in that it further contains an Al(OH)₃ adjuvant.

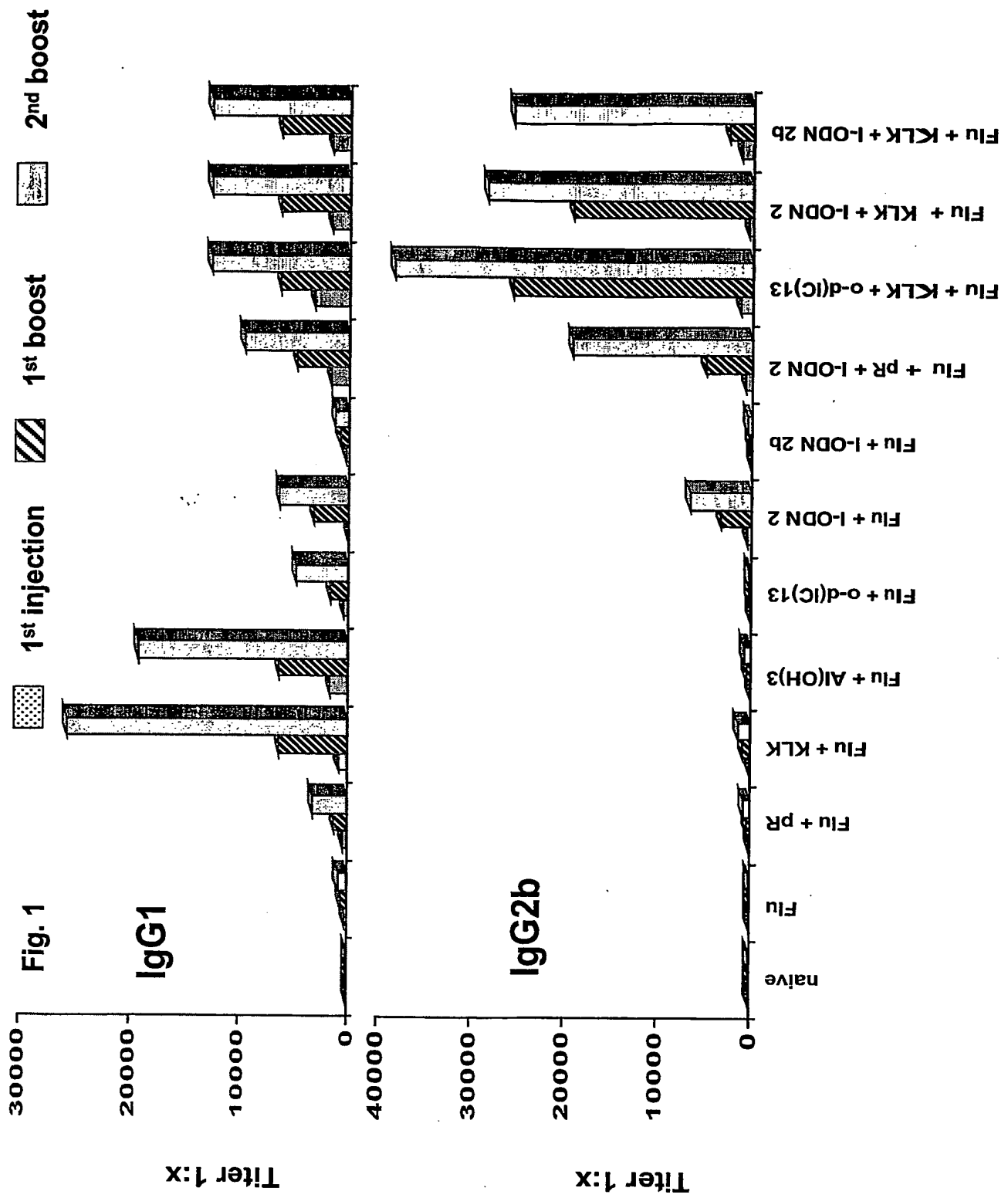
3. Vaccine according to claim 1 or 2, characterised in that said antigen is a viral antigen, preferably an influenza, especially a haemagglutinin antigen or a neuraminidase antigen, HCV or HBV, HIV, HPV or JEV antigen, a combined antigen or a combination of one or more of these antigens.

4. Vaccine according to any one of claims 1 to 3, characterised in that it further contains a polycationic peptide.

5. Vaccine according to any one of claims 1 to 4, characterised in that said Peptide A is KLKL₅KLK and said I-/U-ODN is oligo d(IC)₁₃.

6. Vaccine according to any one of claims 1 to 5, characterised in that it further contains an oligodeoxynucleotide containing a CpG-motif.
7. Vaccine according to any one of claims 1 to 6, characterised in that it further contains a polycationic peptide and an oligodeoxynucleotide containing a CpG-motif.
8. Use of a combination of Peptide A and a I-/U-ODN, both as defined in claim 1, to improve the protective efficacy of a vaccine against viral infection, especially against an infection with influenza virus, HBV, HCV, HPV, HIV or JEV.
9. Use of a combination of Peptide A and a I-/U-ODN, both as defined in claim 1, to improve the antigen-specific type 1 response, especially IgG2-antibody response or IFN-gamma response, of a vaccine against viral infections, especially infections with influenza virus, HBV, HCV, HIV, HPV or JEV, and at the same time preserving or preferably also increasing the type 2 response, especially IgG1-antibody response or interleukin 4 (IL 4) response, of said vaccine.

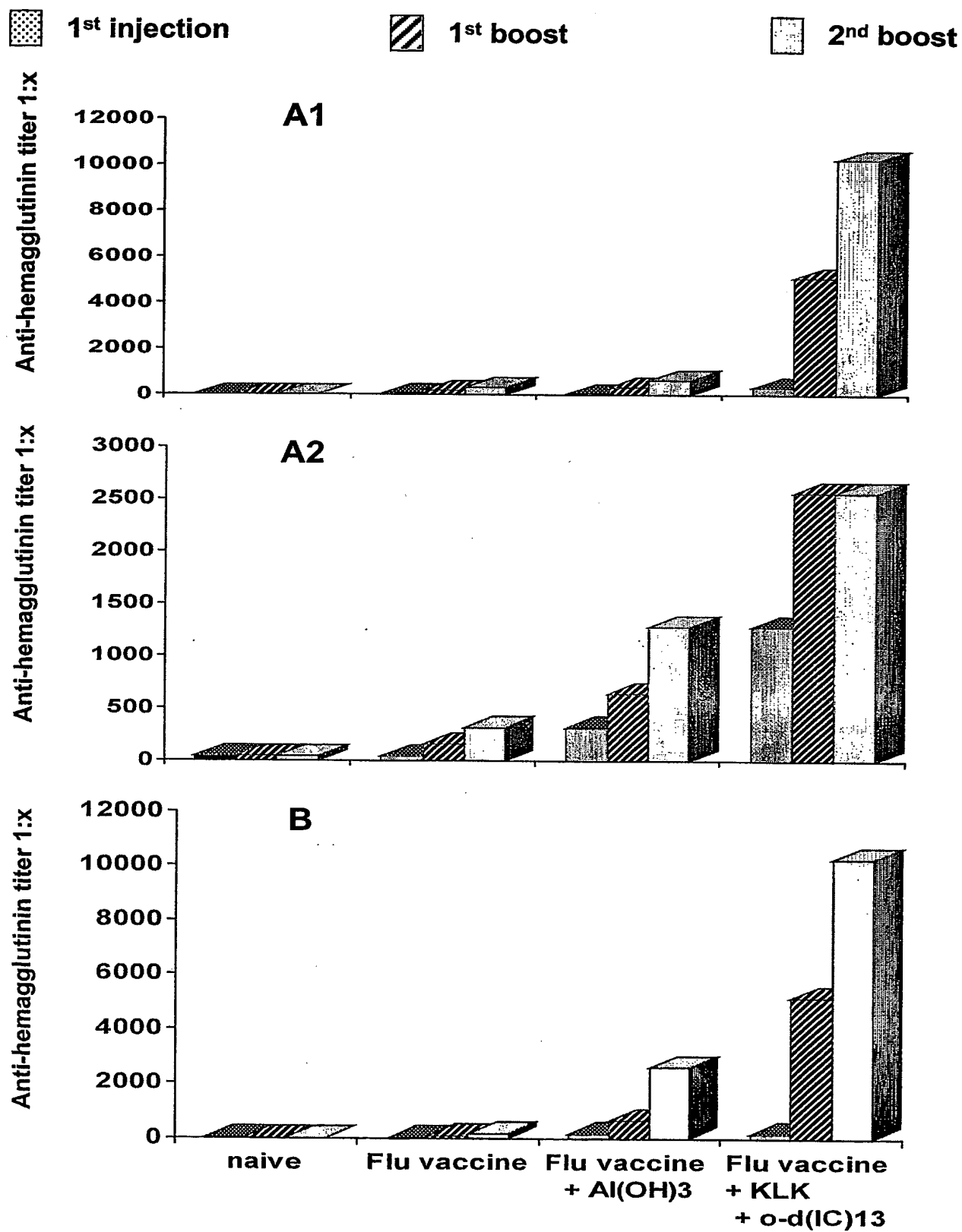
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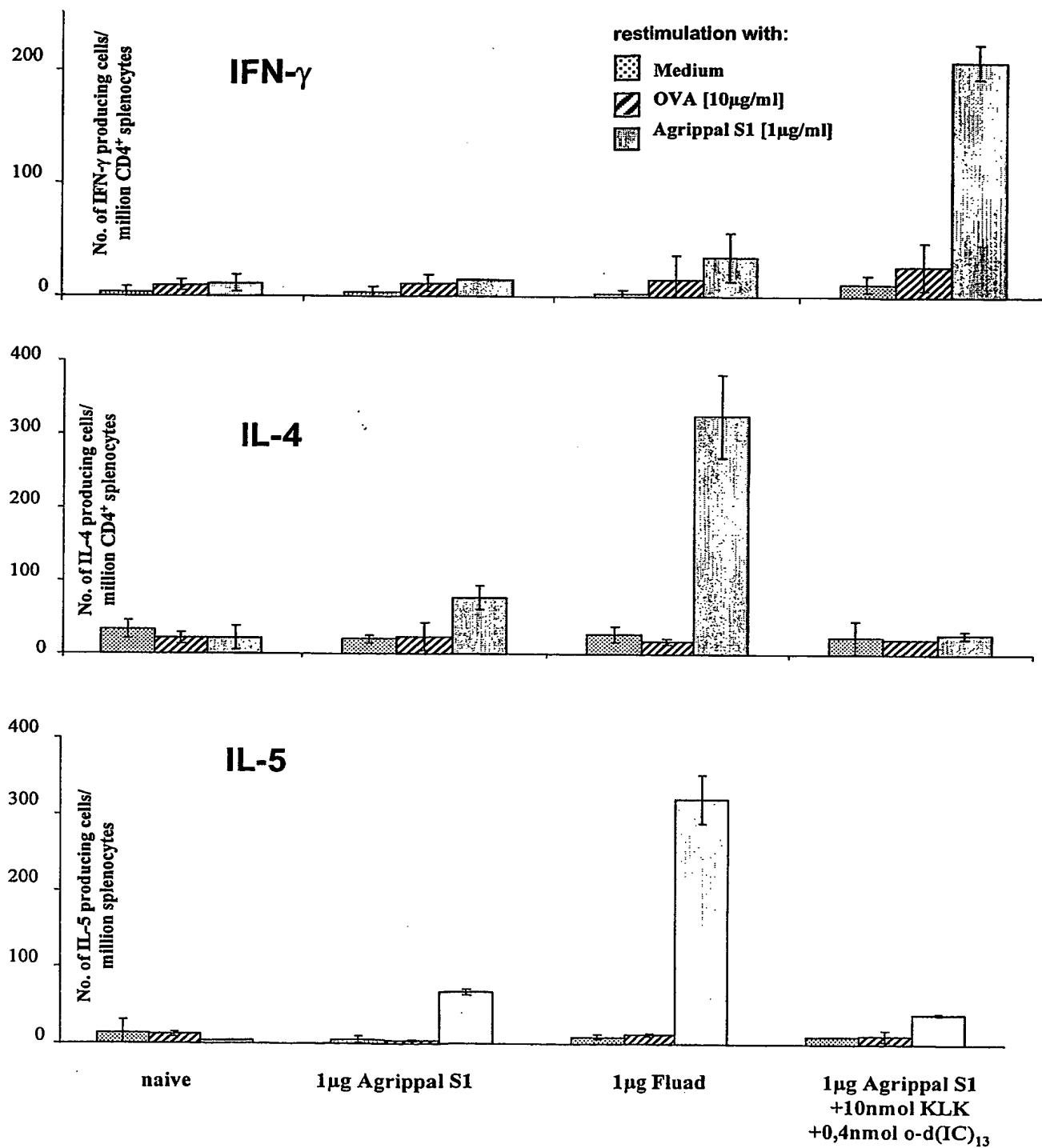
Fig. 2



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Fig. 3a:



BALB/c mice immunized with

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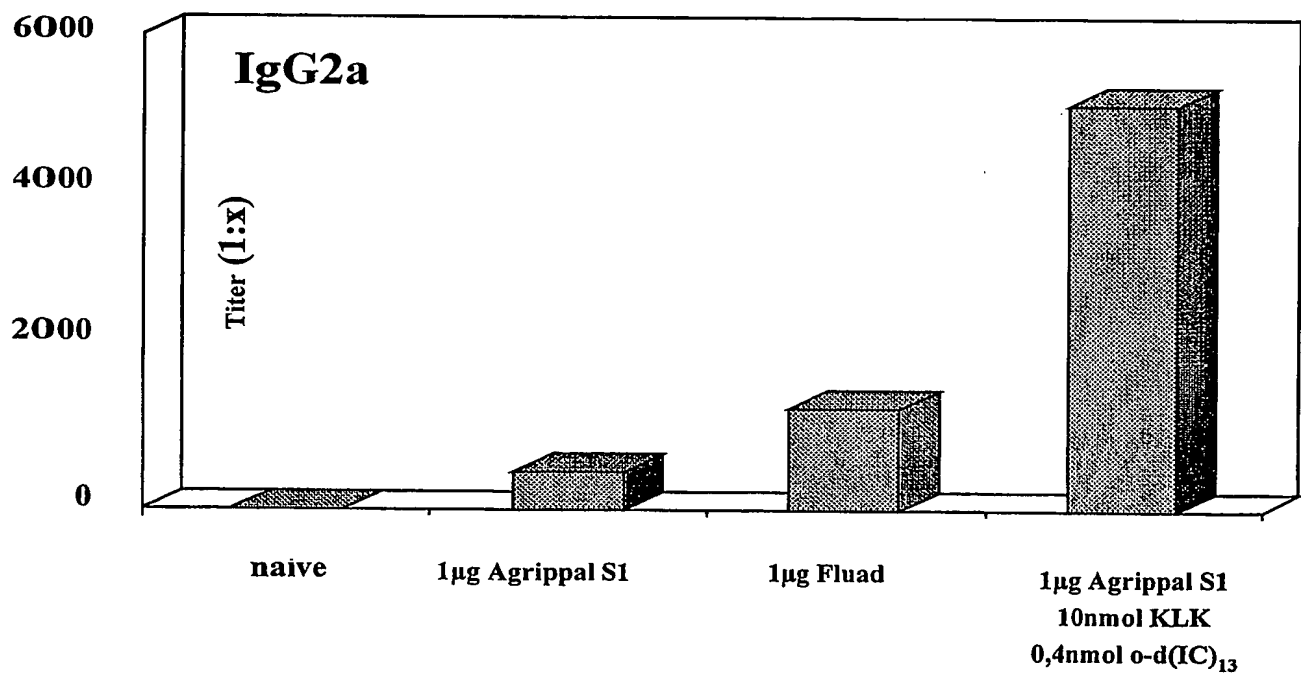
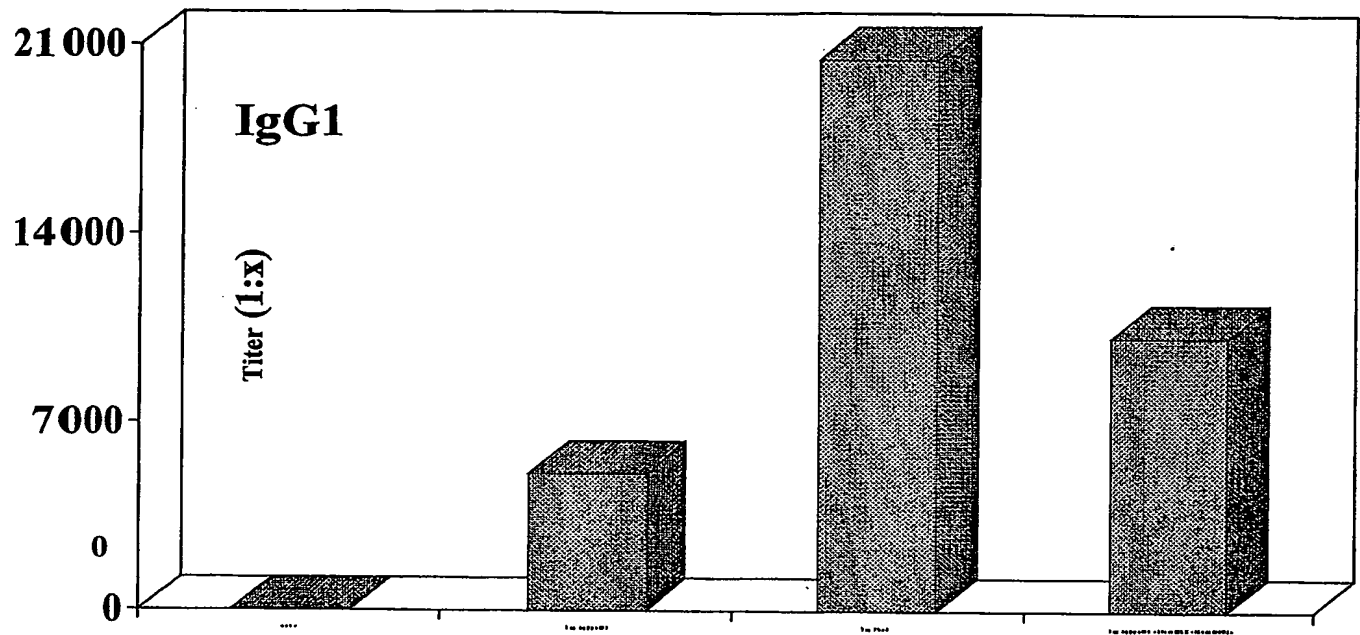
4/14

Fig. 3b:

ELISA-plate coated with:



1µg/ml Agrippal S1



BALB/c mice immunized with

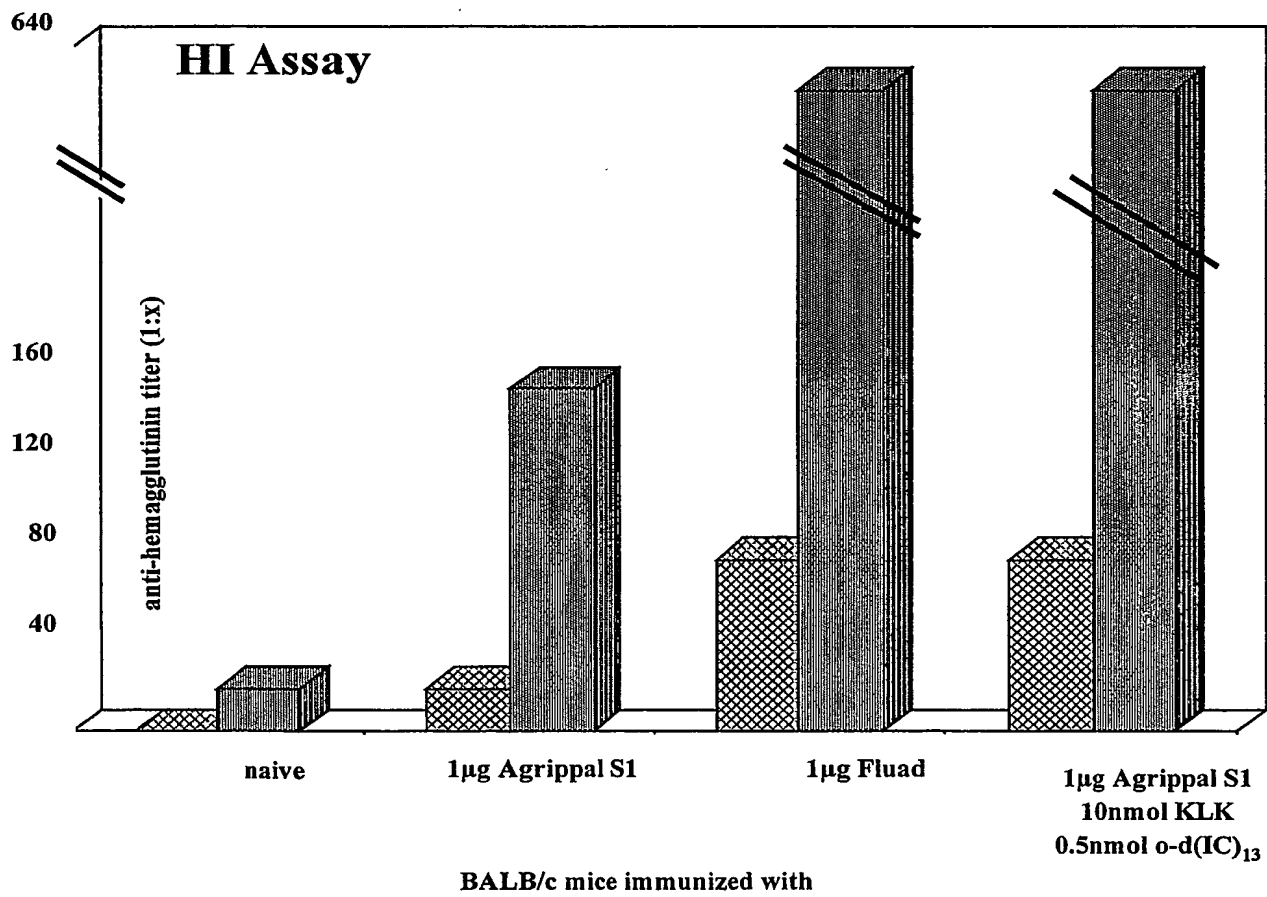
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Fig. 4:

mouse sera tested against hemagglutinin
derived from strain:

- ☒ A/New Caledonia/20/99 (H1/N1)
▨ A/Panama/2007/99 (H3/N2)

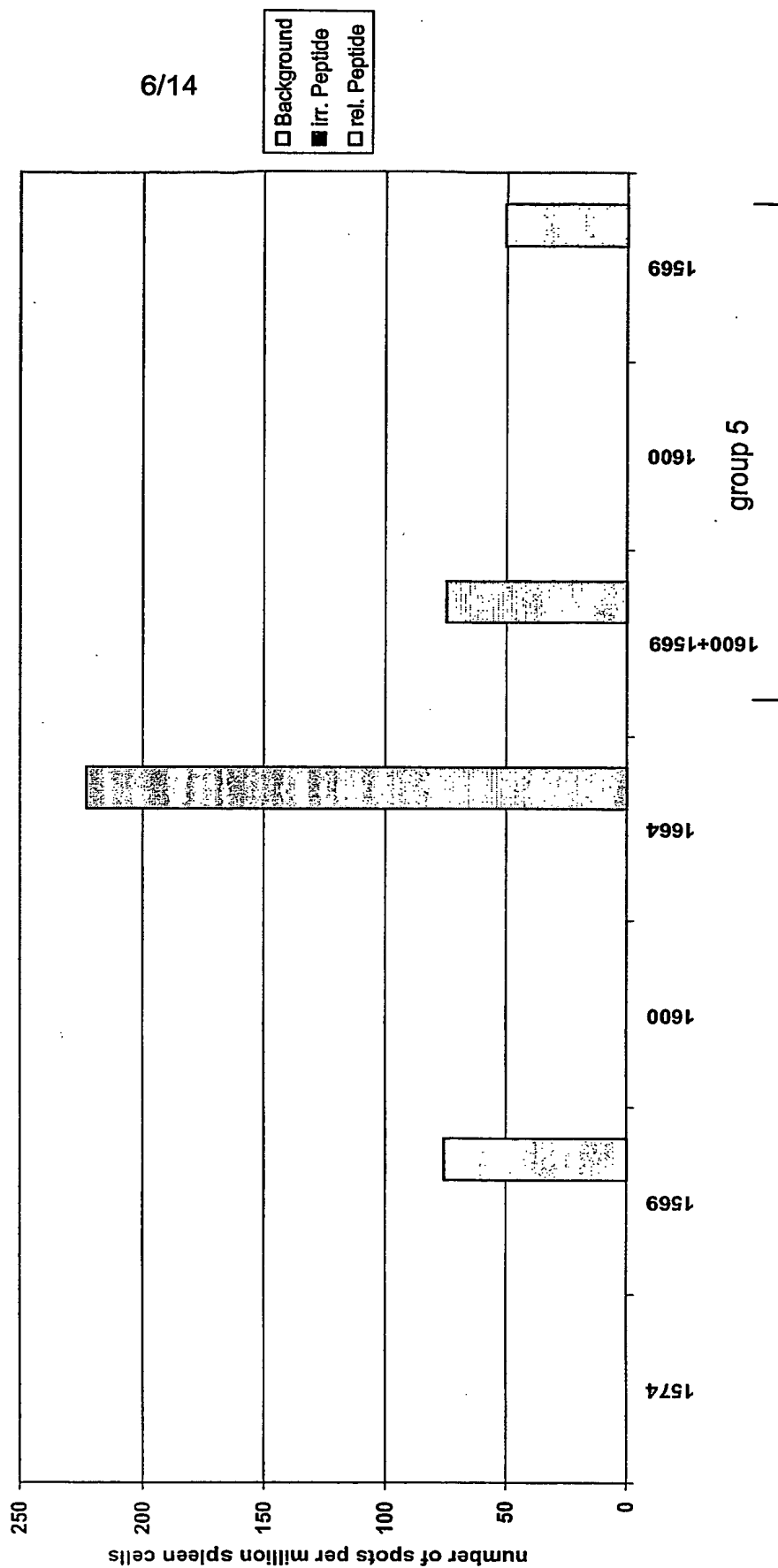


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Fig. 5a:

C57BL/6 mice vaccinated with ' peptide + KLK(o-d(IC)13

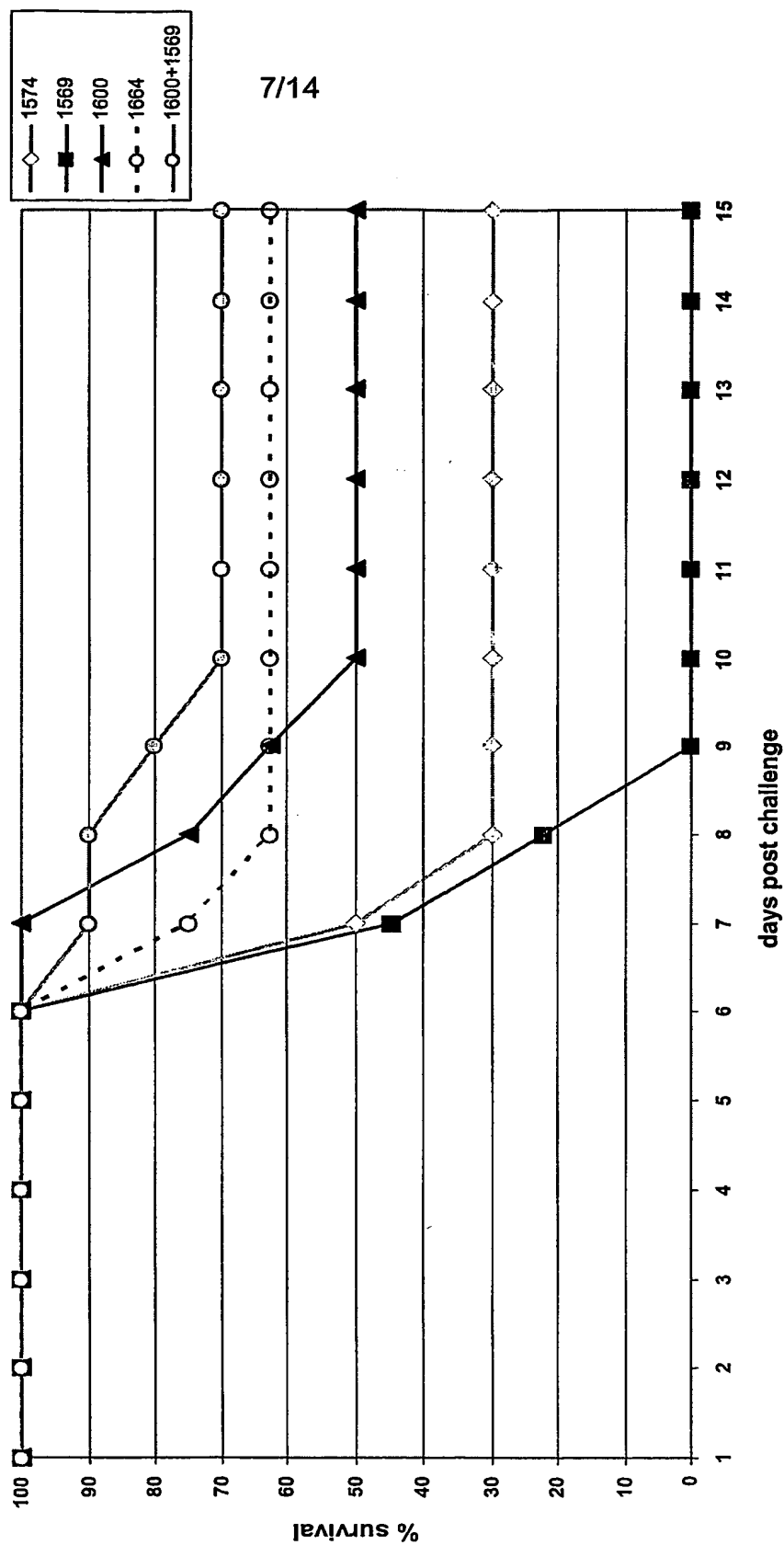
IFNg ELISpot



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Fig. 5b:

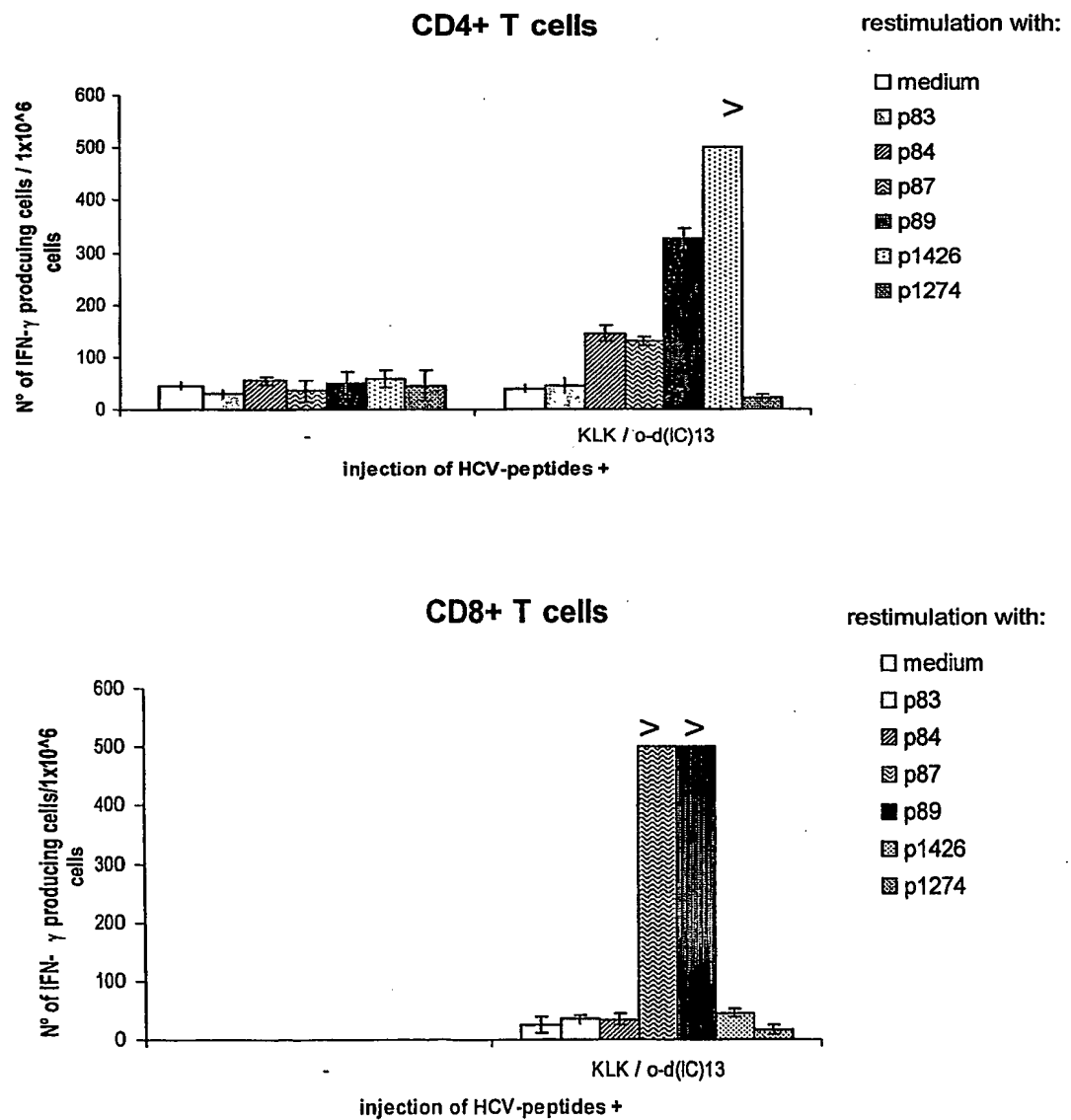
% survival of challenged C57BL/6 mice



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Fig. 6:



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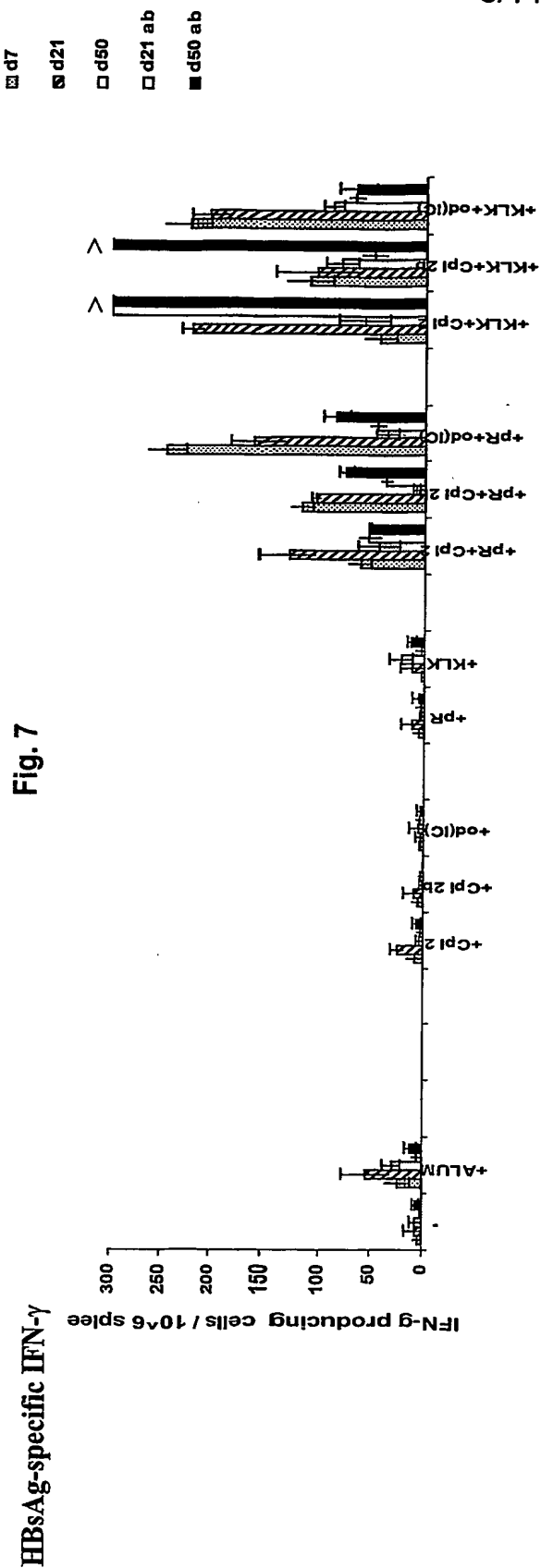
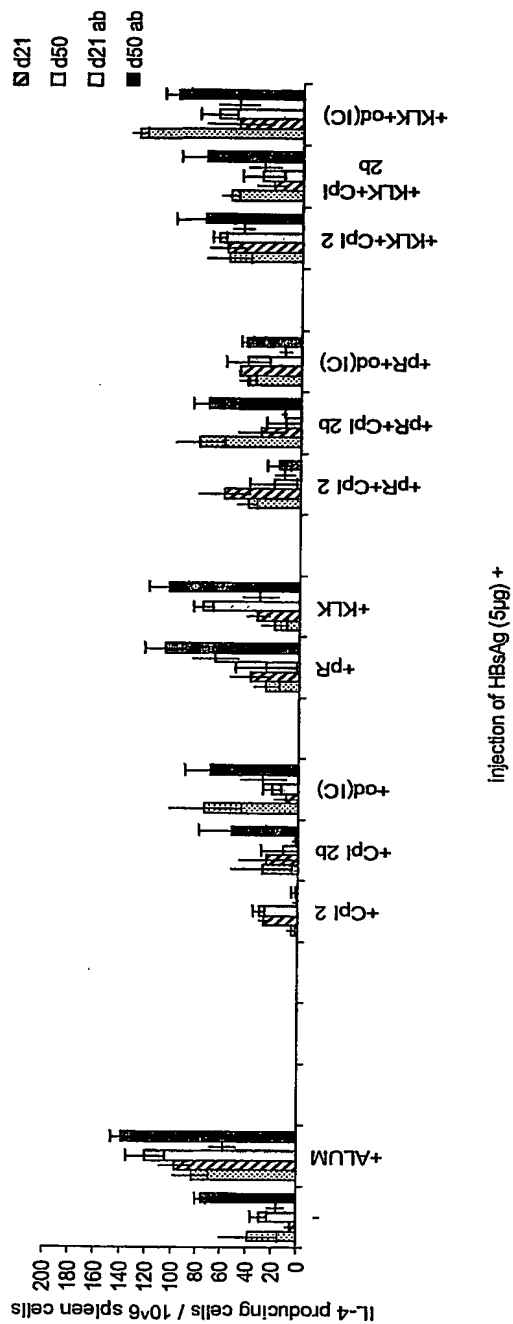


Fig. 7

Injection of HBsAg (5 μ g) +

HBsAg-specific IL-4

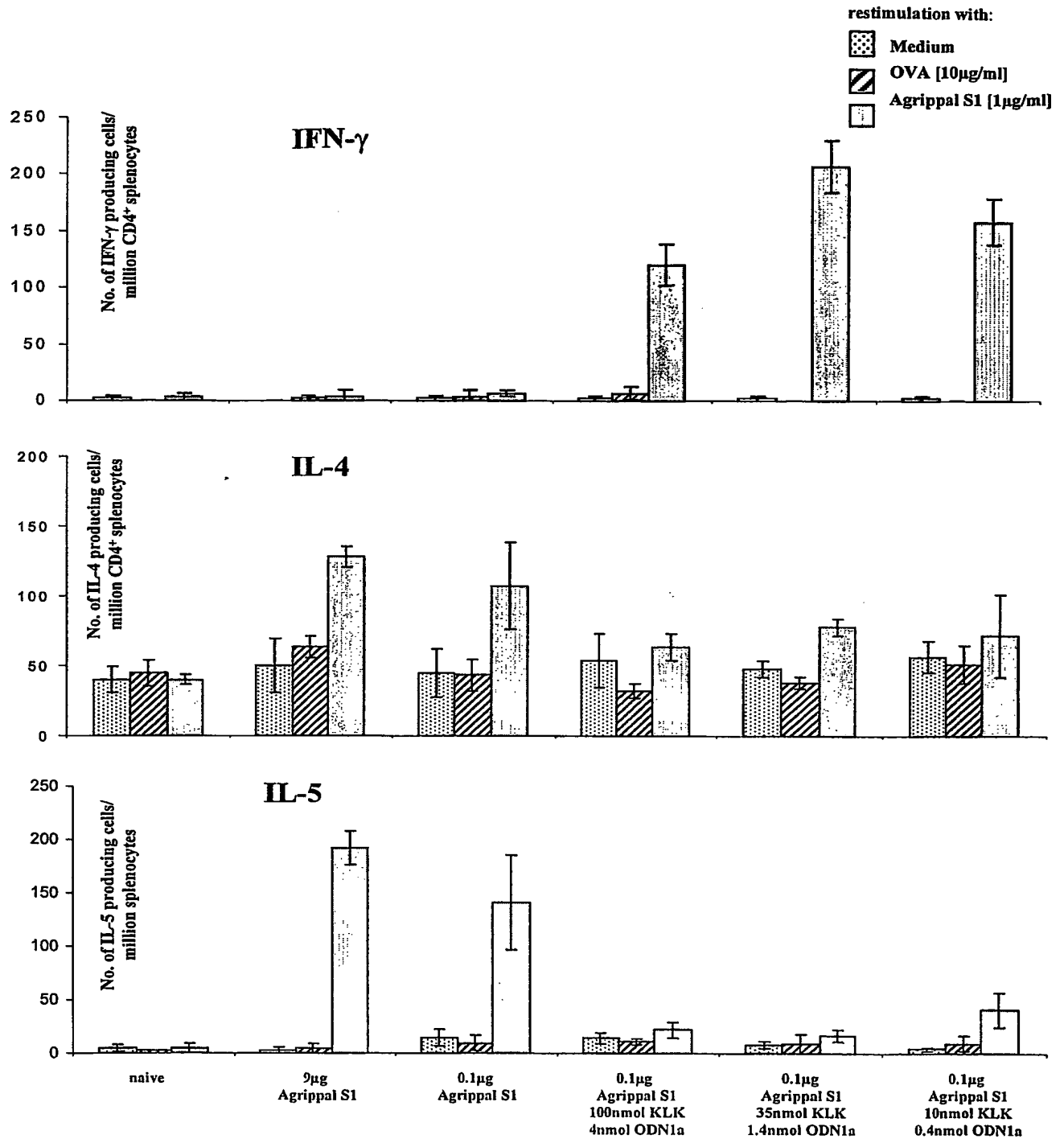


Injection of HBsAg (5 μ g) +

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Fig. 8a:

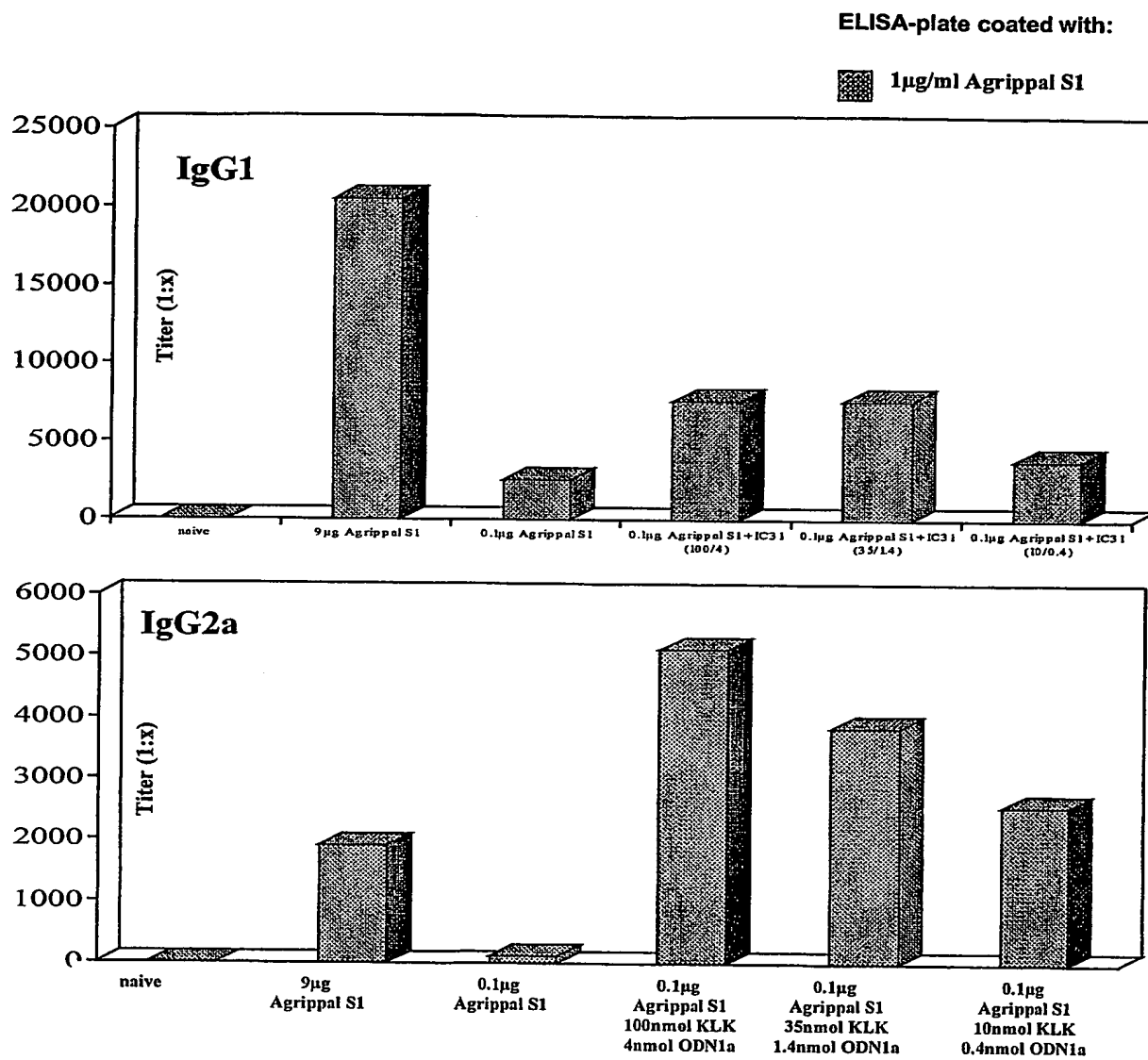


BALB/c mice immunized with

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Fig. 8b:

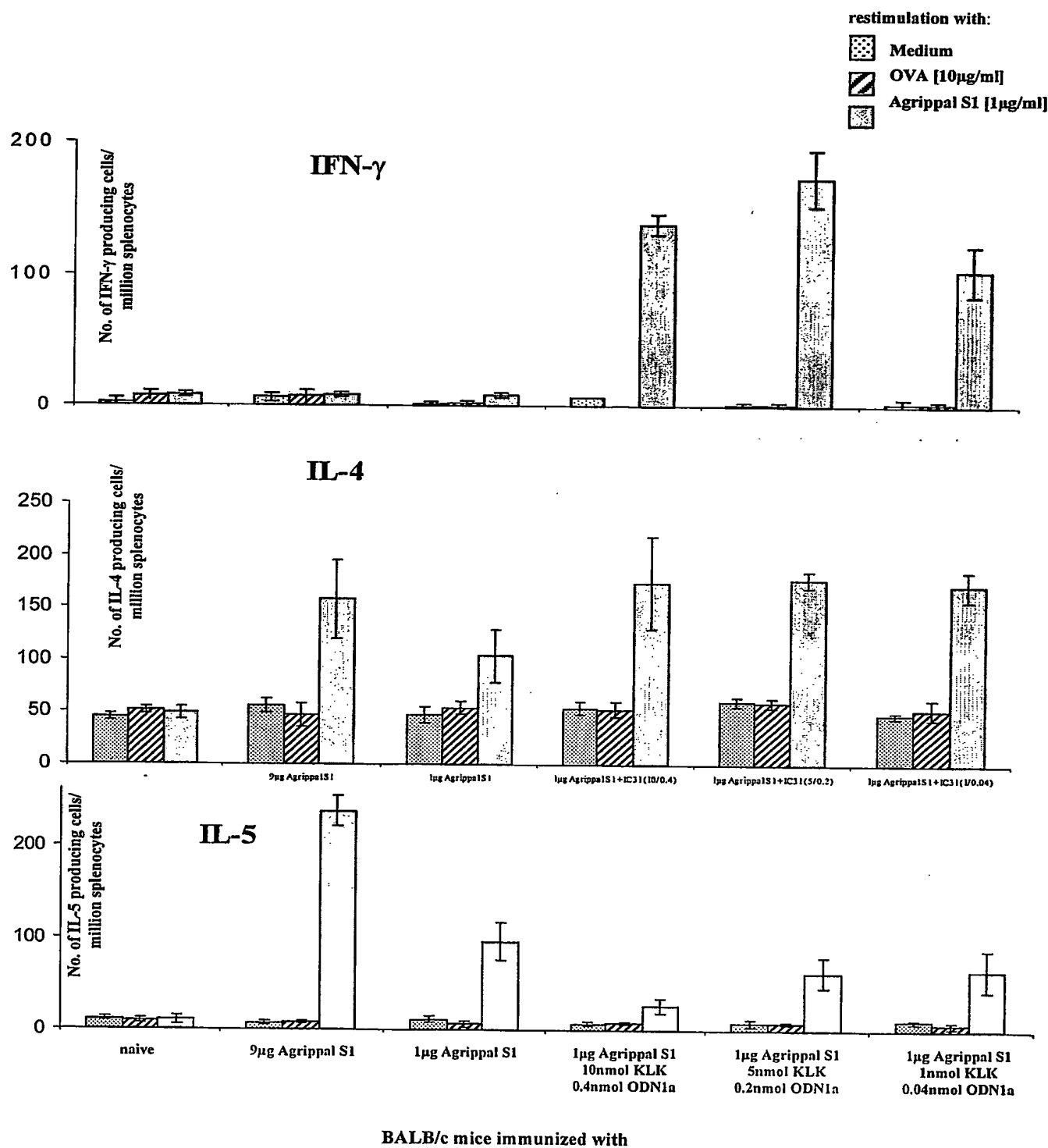


BALB/c mice immunized (i.m.) with

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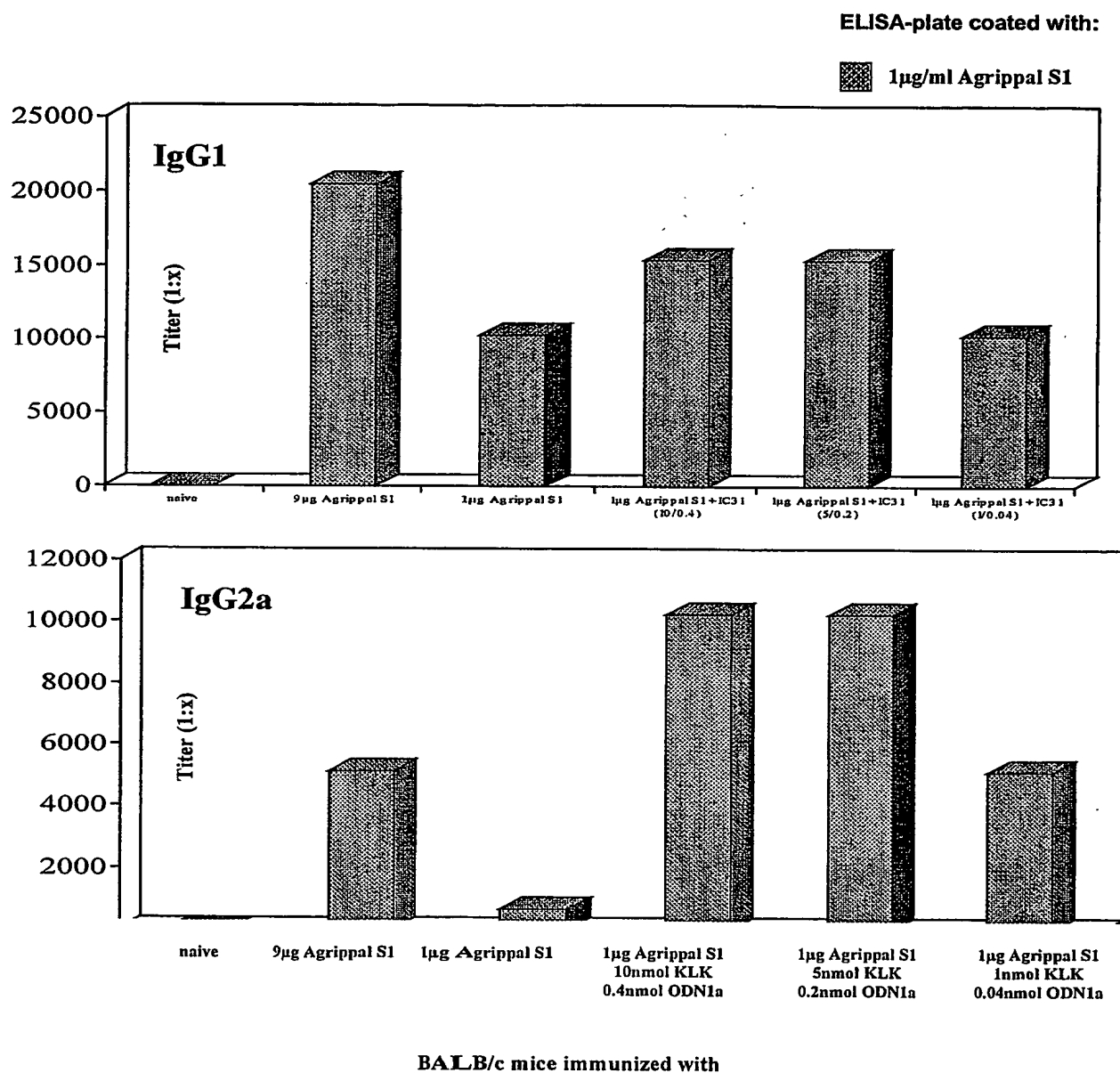
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Fig. 9a:



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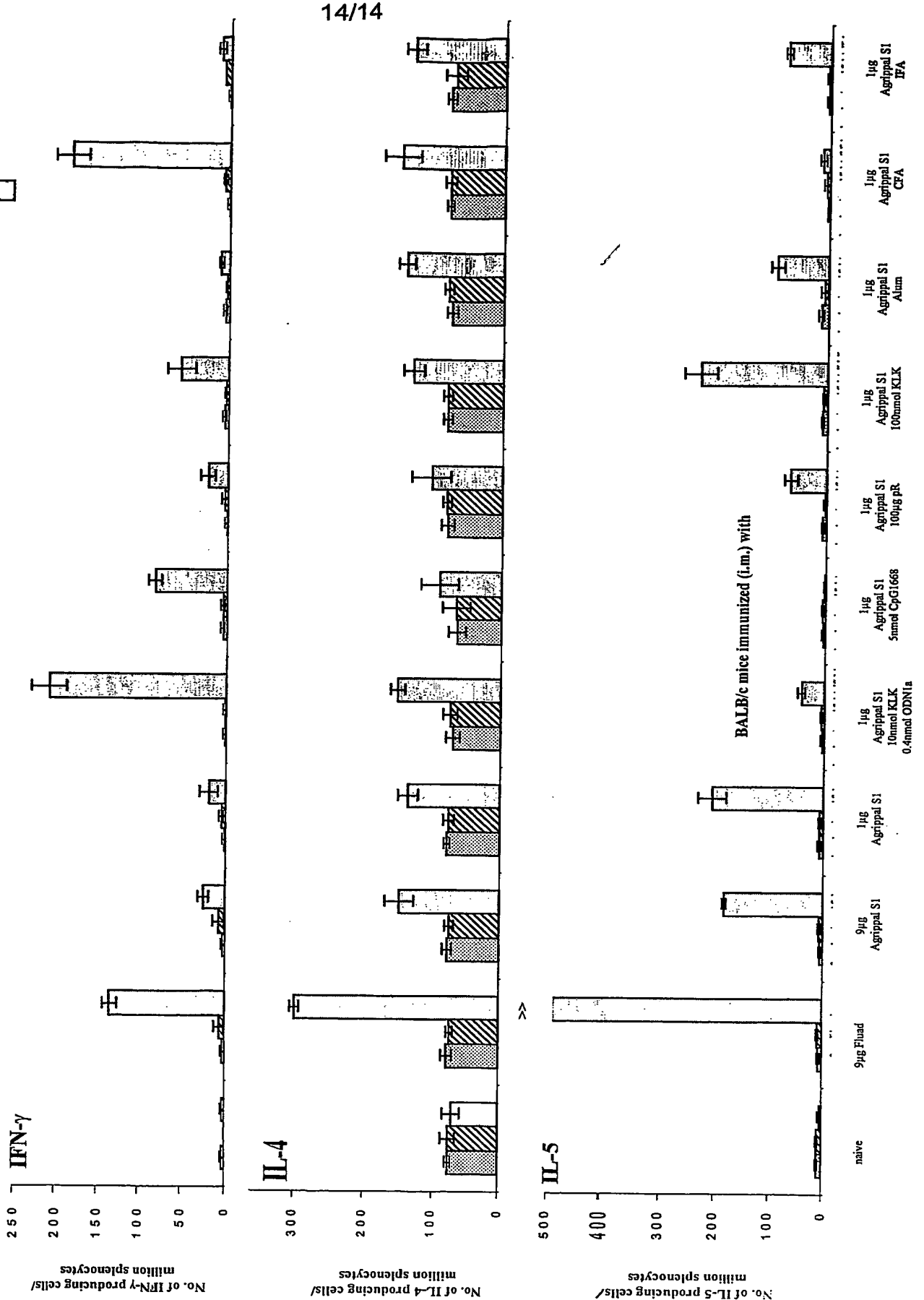
Fig. 9b:



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ALY 11 11 11

Fig. 10



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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/003002

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/39 A61P31/00 A61K39/12 A61K39/29 A61K38/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/93905 A (CISTEM BIOTECHNOLOGIES GMBH ; EGYED ALENA (AT); LINGNAU KAREN (AT); SC) 13 December 2001 (2001-12-13) examples 1-6 page 11, line 2 - line 9 page 13, paragraph 4	1,3,6,8, 9
Y	the whole document	2,5
X	WO 01/93903 A (CISTEM BIOTECHNOLOGIES GMBH ; EGYED ALENA (AT); LINGNAU KAREN (AT); MA) 13 December 2001 (2001-12-13) examples 1-8 page 4, paragraph 4	1,3,8,9

-/-

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search

22 July 2004

Date of mailing of the international search report

06/08/2004

Name and mailing address of the ISA

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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	MCCLUSKIE MICHAEL J ET AL: "Parenteral and mucosal prime-boost immunization strategies in mice with hepatitis B surface antigen and CpG DNA" FEMS IMMUNOLOGY AND MEDICAL MICROBIOLOGY, vol. 32, no. 3, 18 February 2002 (2002-02-18), pages 179-185, XP002288884 ISSN: 0928-8244 the whole document	2
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2004/003002

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 8,9 (each partly)
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 8 and 9 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP2004/003002

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INTERNATIONAL SEARCH REPORT

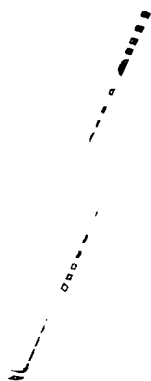
Information on patent family members

International Application No

PCT/EP2004/003002

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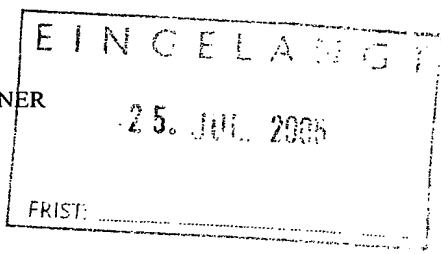
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From the INTERNATIONAL BUREAU

PCTNOTIFICATION CONCERNING
SUBMISSION OR TRANSMITTAL
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

Date of mailing (day/month/year) 14 July 2005 (14.07.2005)		To: 	
Applicant's or agent's file reference R 42928			
International application No. PCT/EP2004/003002		International filing date (day/month/year) 22 March 2004 (22.03.2004)	
International publication date (day/month/year) 07 October 2004 (07.10.2004)		Priority date (day/month/year) 24 March 2003 (24.03.2003)	
Applicant INTERCELL AG et al			

IMPORTANT NOTIFICATION

- By means of this Form, which replaces any previously issued notification concerning submission or transmittal of priority documents, the applicant is hereby notified of the date of receipt by the International Bureau of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to a date of receipt, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
- (If applicable)* The letters "NR" appearing in the right-hand column denote a priority document which, on the date of mailing of this Form, had not yet been received by the International Bureau under Rule 17.1(a) or (b). Where, under Rule 17.1(a), the priority document must be submitted by the applicant to the receiving Office or the International Bureau, but the applicant fails to submit the priority document within the applicable time limit under that Rule, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
- (If applicable)* An asterisk (*) appearing next to a date of receipt, in the right-hand column, denotes a priority document **submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b)** (the priority document was received after the time limit prescribed in Rule 17.1(a) or the request to prepare and transmit the priority document was submitted to the receiving Office after the applicable time limit under Rule 17.1(b)). Even though the priority document was not furnished in compliance with Rule 17.1(a) or (b), the International Bureau will nevertheless transmit a copy of the document to the designated Offices, for their consideration. In case such a copy is not accepted by the designated Office as the priority document, Rule 17.1(c) provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
24 March 2003 (24.03.2003)	03450072.8	EP	11 July 2005 (11.07.2005)
11 April 2003 (11.04.2003)	03450084.3	EP	11 July 2005 (11.07.2005)
11 July 2003 (11.07.2003)	03450171.8	EP	11 July 2005 (11.07.2005)

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